Duplicate volume

THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER
MEMORIAL FUND

EDITED FOR THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

EDITORIAL BOARD

RUDOLPH J. ANDERSON
REGINALD M. ARCHIBALD
W. MANSFIELD CLARK
HANS T. CLARKE
CARL F. CORI
EDWARD A. DOISY
JOSEPH S. FRUTON
A. BAIRD HASTINGS
HOWARD B. LEWIS
ELMER V. McCOLLUM
WILLIAM C. ROSE
WILLIAM C. STADIE
EDWARD L. TATUM
DONALD D. VAN SLYKE
HUBERT B. VICKERY
VINCENT DU VIGNEAUD

VOLUME 176
BALTIMORE
1948

Copyright, 1948
BY
THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC.

PUBLISHED AT YALE UNIVERSITY FOR
THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC.
WAVERLY PRESS, Inc.
Baltimore 2, U. S. A.

CONTENTS OF VOLUME 176

No. 1, October, 1948

PAGE

Peterson, Jack, Harmon, Kent M., and Niemann, Carl. The dependence of the specific activity of urease upon the apparent absolute enzyme concentration 1

Smith, Emil L. Studies on dipeptidases. II. Some properties of the glycyl-L-leucine dipeptidases of animal tissues 9

Smith, Emil L. Studies on dipeptidases. III. Hydrolysis of methylated peptides; the rôle of cobalt in the action of glycylglycine dipeptidase 21

Stekol, Jakob A. The synthesis of S-benzylthiopyruvic acid and its conversion to N-acetyl-S-benzyl-L-cysteine in the rat; unavailability of thiopyruvic acid to rats for growth purposes 33

MacLeod, Robert A., and Snell, Esmond E. The effect of related ions on the potassium requirement of lactic acid bacteria 39

Lester, David, and Greenberg, Leon A. A simple apparatus for the serial measurement of the respiratory exchange in the rat 53

Horn, Millard J., Jones, D. Breese, and Blum, Amos E. Microbiological determination of arginine in proteins and foods 59

Jones, D. Breese, Caldwell, Alvin, and Horn, Millard J. The availability of DL-lanthionine for the promotion of growth in young rats when added to a cystine- and methionine-deficient diet 65

Farlow, M. W. A new synthesis of cystine 71

Munson, Paul L., Jones, Mary Ellen, McCall, Philip J., and Gallagher, T. F. A colorimetric method for estimation of dehydroisoandrosterone and its application to urine extracts 73

Aldous, J. G. The effect of pH upon the toxicity of iodoacetic acid to yeast cells 83

Sheffner, A. Leonard, Kirsner, Joseph B., and Palmer, Walter L. Studies on amino acid excretion in man. II. Amino acids in feces 89

Grafflin, Allan L., and Green, D. E. Studies on the cyclophorase system. II. The complete oxidation of fatty acids 95

Knox, W. Eugene, Noyce, B. N., and Auerbach, V. H. Studies on the cyclophorase system. III. Obligatory sparking of fatty acid oxidation 117

Atchley, William A. Studies on the cyclophorase system. IV. Direct demonstration of β oxidation 123

Trager, William. Further studies on a fat-soluble material from plasma having biotin activity 133

Wilbur, Karl M., and Anderson, Norman G. Electrometric and colorimetric determination of carbonic anhydrase 147

Frieden, Earl, and Winzler, Richard J. The thyroxine-like activity of compounds structurally related to thyroxine 155

Sauberlich, H. E., and Baumann, C. A. A factor required for the growth of *Leuconostoc citrovorum* 165

Wilhelmi, Alfred E., and Sayers, George. Corrections of published electrophoretic mobilities of adrenocorticotropic and parathyroid hormones 175

Follett, Archie E. Preparation and some properties of hyaluronic acid from umbilical cord of the pig 177

LEVY, MILTON, and YOUNG, NELSON F. Chemistry of the chick embryo. VI. Accumulation of diphosphopyridine nucleotide 185
ZILVERSMIT, D. B., ENTENMAN, C., and CHAIKOFF, I. L. The measurement of turnover of the various phospholipides in liver and plasma of the dog and its application to the mechanism of action of choline 193
ZILVERSMIT, D. B., ENTENMAN, C., and CHAIKOFF, I. L. The turnover rates of plasma lecithin and plasma sphingomyelin as measured by the disappearance of their radioactive phosphorus from the circulation 209
ROBERTS, EUGENE. Estimation of arginase activity in homogenates 213
FITZGERALD, ROBERT J. The inactivation of streptomycin by cyanate 223
MATHEWS, MARTIN B. The reduction of cozymase by sodium borohydride 229
STUMPF, P. K. Carbohydrate metabolism in higher plants. I. Pea aldolase.... 233
KOCHAKIAN, CHARLES D., and BARTLETT, MARY N. The effect of crystalline adrenal cortical steroids, DL-thyroxine, and epinephrine on the alkaline and acid phosphatases and arginase of the liver and kidney of the normal adult rat 243
ROTH, LLOYD J., LEIFER, EDGAR, HOGNESS, JOHN R., and LANGHAM, WRIGHT H. Studies on the metabolism of radioactive nicotinic acid and nicotinamide in mice 249
SCHNEIDER, WALTER C. Intracellular distribution of enzymes. III. The oxidation of octanoic acid by rat liver fractions 259
JORPES, J. ERIK, and GARDELL, SVEN. On heparin monosulfuric acid 267
JORPES, J. ERIK, WERNER, BIRGITTA, and ÅBERG, BERTIL. The fuchsin-sulfurous acid test after periodate oxidation of heparin and allied polysaccharides 277
SCOTT, KENNETH G., AXELROD, DOROTHY J., FISHER, HARVEY, CROWLEY, J. F., and HAMILTON, JOSEPH G. The metabolism of plutonium in rats following intramuscular injection. Plates 1 and 2 283
AXELROD, BERNARD. A study of the mechanism of "phosphotransferase" activity by use of radioactive phosphorus 295
GEST, HOWARD, and KAMEN, MARTIN D. Studies on the phosphorus metabolism of green algae and purple bacteria in relation to photosynthesis 299
ALTMAN, KURT I., CASARETT, G. W., MASTERS, R. E., NOONAN, T. R., and SALOMON, K. Hemoglobin synthesis from glycine labeled with radioactive carbon in its α-carbon atom 319
LEVEDAHL, BLAINE H., and SAMUELS, LEO T. The effect of testosterone and methyltestosterone on guanidoacetic acid, creatine, and creatinine in plasma and urine 327
STEIN, WILLIAM H., and MOORE, STANFORD. Chromatography of amino acids on starch columns. Separation of phenylalanine, leucine, isoleucine, methionine, tyrosine, and valine 337
MOORE, STANFORD, and STEIN, WILLIAM H. Photometric ninhydrin method for use in the chromatography of amino acids 367
ZILVERSMIT, D. B., CHAIKOFF, I. L., FELLER, D. D., and MASORO, E. J. Oxidation of glucose labeled with radioactive carbon by normal and alloxan-diabetic rats 389
PUTNAM, FRANK W., LAMANNA, CARL, and SHARP, D. G. Physicochemical properties of crystalline *Clostridium botulinum* type A toxin 401
THORNE, C. B., and PETERSON, W. H. Xanthomycins A and B, new antibiotics produced by a species of *Streptomyces* 413

MacFarland, M. L., and McHenry, E. W. Further observations on the lipotropic need for inositol........ 429
Vallee, Bert L., and Gibson, John G., 2nd. An improved dithizone method for the determination of small quantities of zinc in blood and tissue samples........ 435
Vallee, Bert L., and Gibson, John G., 2nd. The zinc content of normal human whole blood, plasma, leucocytes, and erythrocytes........ 445

Letters to the Editors

Kaplan, Nathan O., and Lipmann, Fritz. The acetyl precursor in pyruvate synthesis in *Escherichia coli*........ 459
Stimmel, Benjamin F., Grollman, Arthur, and Huffman, Max N. The conversion of 16-ketoestrone to estriol *in vivo*........ 461
Korkes, Seymour, and Ochoa, Severo. Adaptive conversion of malate to lactate and carbon dioxide by *Lactobacillus arabinosus*........ 463
Schwimmer, Sigmund, and Balls, A. K. Crystalline α-amylase from barley malt........ 465

No. 2, November, 1948

Hankes, L. V., Riesen, W. H., Henderson, L. M., and Elvehjem, C. A. Liberation of amino acids from raw and heated casein by acid and enzyme hydrolysis........ 467
Lightfoot, Lois H., and Coolidge, Thomas B. The distribution of collagen in the guinea pig........ 477
Witter, Robert F., and Stotz, Elmer. Synthesis and properties of triacetic acid........ 485
Witter, Robert F., Snyder, Jeanette, and Stotz, Elmer. Colorimetric determination of acetylacetone and related β-diketones........ 493
Witter, Robert F., and Stotz, Elmer. The metabolism *in vitro* of triacetic acid and related diketones........ 501
MacVicar, Robert, and Burris, R. H. Studies on nitrogen metabolism in tomato with use of isotopically labeled ammonium sulfate........ 511
Brickson, W. L., Henderson, L. M., Solhjell, I., and Elvehjem, C. A. Antagonism of amino acids in the growth of lactic acid bacteria........ 517
Moubasher, Radwan. Reaction of *peri*-naphthindan-2,3,4-trione hydrate with *l*-ascorbic acid and other enediol compounds, with a note on the isolation of dehydro-*l*-ascorbic acid........ 529
Mueller, G. C., and Miller, J. A. The metabolism of 4-dimethylaminoazobenzene by rat liver homogenates........ 535
Siplet, Herman, Komarov, S. A., and Shay, Harry. The estimation of mucin in gastric juice........ 545
Bliss, Alfred F. The absorption spectra of visual purple of the squid and its bleaching products........ 563
Cartwright, George E., and Wintrobe, Maxwell M. Studies on free erythrocyte protoporphyrin, plasma copper, and plasma iron in protein-deficient and iron-deficient swine........ 571
Cartwright, George E., Smith, Emil L., Brown, Douglas M., and Wintrobe, Maxwell M. Electrophoretic analyses of sera of normal and hypoproteinemic swine........ 585

KIELLEY, W. WAYNE, and MEYERHOF, OTTO. Studies on adenosinetriphosphatase of muscle. II. A new magnesium-activated adenosinetriphosphatase 591

JEANES, ALLENE, WILHAM, C. A., and MIERS, J. C. Preparation and characterization of dextran from *Leuconostoc mesenteroides* 603

JEANES, ALLENE, SCHIELTZ, N. C., and WILHAM, C. A. Molecular association in dextran and in branched amylaceous carbohydrates. Plate 3 617

UMBREIT, W. W., O'KANE, D. J., and GUNSALUS, I. C. Function of the vitamin B_6 group: mechanism of transamination 629

TAUROG, ALVIN, and CHAIKOFF, I. L. The nature of the circulating thyroid hormone 639

JANSEN, EUGENE F. The isolation and identification of 2,2′-dithiolisobutyric acid from asparagus 657

KORNBERG, ARTHUR, and LINDBERG, OLOV. Diphosphopyridine nucleotide pyrophosphatase 665

HORN, MILLARD J., JONES, D. BREESE, and BLUM, AMOS E. Microbiological determination of phenylalanine in proteins and foods 679

PETERMANN, MARY L., and LAMB, CATHERINE M. The nucleohistone of beef spleen 685

AXELROD, A. E., PURVIS, SARAH ELLEN, and HOFMANN, KLAUS. The application of structural analogues to enzymatic studies. I. Studies on the mode of action of biotin analogues 695

VISCHER, ERNST, and CHARGAFF, ERWIN. The separation and quantitative estimation of purines and pyrimidines in minute amounts 703

VISCHER, ERNST, and CHARGAFF, ERWIN. The composition of the pentose nucleic acids of yeast and pancreas 715

WILHELMI, ALFRED E., FISHMAN, JACOB B., and RUSSELL, JANE A. A new preparation of crystalline anterior pituitary growth hormone 735

BOWEN, WILLIAM J. Notes on myoglobin preparation and iron content 747

ROSE, WILLIAM C., OESTERLING, M. JANE, and WOMACK, MADELYN. Comparative growth on diets containing ten and nineteen amino acids, with further observations upon the rôle of glutamic and aspartic acids 753

HEPPEL, LEON A., and PORTERFIELD, VIRGINIA T. Enzymatic dehalogenation of certain brominated and chlorinated compounds 763

MAZUR, ABRAHAM, and SHORR, EPHRAIM. Hepatorenal factors in circulatory homeostasis. IX. The identification of the hepatic vasodepressor substance, VDM, with ferritin 771

DUBNOFF, JACOB W., and BORSOOK, HENRY. Dimethylthetin and dimethyl-β-propiothetin in methionine synthesis 789

DEUTSCH, H. F., and NICHOL, J. C. Biophysical studies of blood plasma proteins. X. Fractionation studies of normal and immune horse serum 797

MILNER, HAROLD W. The fatty acids of *Chlorella* 813

FELS, I. GORDON, and CHELDELIN, VERNON H. Methionine in selenium poisoning 819

SRERE, PAUL A., CHAIKOFF, I. L., and DAUBEN, W. G. The *in vitro* synthesis of cholesterol from acetate by surviving adrenal cortical tissue 829

SMITH, EMIL L., and SLONIM, N. BALFOUR. The specificity of leucine aminopeptidase 835

LI, CHOH HAO, SIMPSON, MIRIAM E., and EVANS, HERBERT M. Iodination of hypophyseal growth hormone 843

Pearlman, W. H., and Cerceo, Emily. The isolation of pregnanol-3(α)-one-20, pregnanediol-3(α),20(β), and etiocholanediol-3(α),17(β) from the bile of pregnant cows 847

Eadie, G. S., Bernheim, Frederick, and Fitzgerald, Robert J. The kinetics of the oxidation of benzoic acid by certain *Mycobacteria* 857

Beloff, Anne, and Anfinsen, Christian B. The products of proteolysis of some purified proteins 863

Adler, M., and Wintersteiner, O. A reinvestigation of flavacidin, the penicillin produced by *Aspergillus flavis* 873

Carpenter, Frederick H., Turner, Robert A., and du Vigneaud, Vincent. Benzylpenicillenic acid as an intermediate in the synthesis of benzylpenicillin (penicillin G) 893

du Vigneaud, Vincent, Stacy, Gardner W., and Todd, David. The synthesis of DL-β,β-diethylcysteine and DL-β-ethyl-β-methylcysteine 907

Carpenter, Frederick H., Stacy, Gardner W., Genghof, Dorothy S., Livermore, Arthur H., and du Vigneaud, Vincent. The preparation and antibacterial properties of the crude sodium salts of some synthetic penicillins 915

Grisolia, Santiago, and Cohen, Philip P. Study of carbon dioxide fixation in the synthesis of citrulline 929

Hogeboom, George H., and Barry, Guy T. Purification of diphosphopyridine nucleotide by counter-current distribution 935

Artom, Camillo, and Cornatzer, W. E. The action of ethanolamine, methylethanolamine, and dimethylethanolamine on lipide phosphorylation 949

Orten, James M., and Bucciero, Mary C. The effect of cysteine, histidine, and methionine on the production of polycythemia by cobalt 961

Bennett, Edward L., and Niemann, Carl. The use of ion exchange resins in the isolation of blood group A-specific substance from hog gastric mucin 969

Salivar, Charles J., Grenfell, Thomas C., and Brown, Ellis V. Studies on the naturally occurring penicillins. II. Precipitation of crystalline ammonium penicillins 977

Kibrick, Andre C., and Blonstein, Muriel. Fractionation of serum into albumin and α-, β-, and γ-globulin by sodium sulfate 983

Letters to the Editors

Herbst, Edward J., and Snell, Esmond E. Putrescine as a growth factor for *Hemophilus parainfluenzae* 989

Shive, William, Ravel, Joanne Macow, and Harding, W. M. An interrelationship of purines and vitamin B_{12} 991

Meyer, Karl. Highly viscous sodium hyaluronate 993

Sakami, Warwick. The conversion of formate and glycine to serine and glycogen in the intact rat 995

Smith, Emil L., and Hanson, H. Theo. Pancreatic carboxypeptidase; a metal protein 997

No. 3, December, 1948

Chanutin, Alfred, and Ludewig, Stephan. Biochemical studies on the whole and fractionated thymus of rats injected with β-chloroethyl vesicants 999

LePage, G. A. Glycolysis in tumor homogenates 1009

LePage, G. A., and Schneider, Walter C. Centrifugal fractionation of glycolytic enzymes in tissue homogenates 1021

Maw, George A., and du Vigneaud, Vincent. An investigation of the biological behavior of the sulfur analogue of choline 1029

Maw, George A., and du Vigneaud, Vincent. Compounds related to dimethylthetin as sources of labile methyl groups 1037

Behrens, Otto K., and Kingkade, Mary Jane. Biosynthesis of penicillins. VIII. Studies with new biosynthetic penicillins on penicillin resistance 1047

Singal, S. A., Sydenstricker, V. P., and Littlejohn, Julia M. The rôle of tryptophan in the nutrition of dogs on nicotinic acid-deficient diets 1051

Singal, S. A., Sydenstricker, V. P., and Littlejohn, Julia M. Further studies on the effect of some amino acids on the growth and nicotinic acid storage of rats on low casein diets 1063

Singal, S. A., Sydenstricker, V. P., and Littlejohn, Julia M. The nicotinic acid content of tissues of rats on corn rations 1069

Potter, Van R., Pardee, Arthur B., and Lyle, Gloria G. The assay of animal tissues for respiratory enzymes. VIII. Conditions for oxalacetate oxidation in whole homogenates 1075

Pardee, Arthur B., and Potter, Van R. Inhibition of succinic dehydrogenase by oxalacetate 1085

Keith, Cecelia K., Broach, Wilson J., Warren, Doris, Day, Paul L., and Totter, John R. Xanthine oxidase and tyrosinase in the livers of chicks receiving graded levels of dietary pteroylglutamic acid 1095

Sobel, Albert E., and Hanok, Albert. Calcification of teeth. I. Composition in relation to blood and diet 1103

Loring, Hubert S., Ordway, George L., and Pierce, John G. A method of assay for cytidine and uridine by means of a pyrimidine-deficient strain of *Neurospora* 1123

Pierce, John G., and Loring, Hubert S. Purine and pyrimidine antagonism in a pyrimidine-deficient mutant of *Neurospora* 1131

Treadwell, C. R. Growth and lipotropism. II. The effects of dietary methionine, cystine, and choline in the young white rat 1141

Treadwell, C. R. Growth and lipotropism. III. The effect of supplementary cystine, methionine, and choline in low protein diets 1149

Rabinowitz, Jesse C., and Snell, Esmond E. The vitamin B_6 group. XIV. Distribution of pyridoxal, pyridoxamine, and pyridoxine in some natural products 1157

Beck, Jay V. A microbiological method for the determination of adenine 1169

Jager, B. V., Smith, Emil L., Nickerson, Margaret, and Brown, Douglas M. Immunological and electrophoretic studies on human γ-globulins 1177

Albanese, Anthony A., Davis, Virginia I., Lein, Marilyn, and Smetak, Emilie M. The biochemistry of the metabolic fecal protein nitrogen 1189

Richter, Derek, and Dawson, Rex M. C. The ammonia and glutamine content of the brain 1199

Trager, William. Biotin and fat-soluble materials with biotin activity in the nutrition of mosquito larvae 1211

Griffin, A. Clark, Nye, William N., Noda, Lafayette, and Luck, J. Murray. Tissue proteins and carcinogenesis. I. The effect of carcinogenic azo dyes on liver proteins 1225

RAPPORT, MAURICE M., GREEN, ARDA ALDEN, and PAGE, IRVINE H. Serum vasoconstrictor (serotonin). III. Chemical inactivation 1237

RAPPORT, MAURICE M., GREEN, ARDA ALDEN, and PAGE, IRVINE H. Serum vasoconstrictor (serotonin). IV. Isolation and characterization 1243

KRISHNASWAMY, K. G., and SREENIVASAN, A. Separation and determination of the amylose and amylopectin fractions of starch 1253

LIFSON, NATHAN, LORBER, VICTOR, SAKAMI, WARWICK, and WOOD, HARLAND G. The incorporation of acetate and butyrate carbon into rat liver glycogen by pathways other than carbon dioxide fixation 1263

LEATHEM, JAMES H. Influence of testosterone propionate on the plasma and liver proteins of hypothyroid rats 1285

WOOLLEY, D. W. Studies on the structure of lycomarasmin 1291

WOOLLEY, D. W. Synthesis and determination of lycomarasmin activity of some derivatives of aspartic acid 1299

PRUSOFF, W. H., TEPLY, L. J., and KING, C. G. The influence of pteroylglutamic acid on nucleic acid synthesis in *Lactobacillus casei* 1309

McCOY, R. H., McKIBBEN, JULIET N., AXELROD, A. E., and HOFMANN, KLAUS. Oxybiotin metabolism in the chick. I. Deposition of oxybiotin in tissues 1319

McCOY, R. H., McKIBBEN, JULIET N., AXELROD, A. E., and HOFMANN, KLAUS. Oxybiotin metabolism in the chick. II. Oxybiotin and biotin balance studies 1327

ANKER, H. S. Synthesis of carbonyl-labeled pyruvic acid 1333

ANKER, H. S. Some aspects of the metabolism of pyruvic acid in the intact animal 1337

ANKER, H. S., and RAPER, R. On the metabolic fate of pyruvamide and acetamide 1353

HEYMANN, HANS, and FIESER, LOUIS F. Naphthoquinone antimalarials. XXI. Antisuccinate oxidase activity 1359

FIESER, LOUIS F., and HEYMANN, HANS. Naphthoquinone antimalarials. XXII. Relative antirespiratory activities (*Plasmodium lophurae*) 1363

SEIFTER, SAM, HARKNESS, DAVID M., RUBIN, LEONARD, and MUNTWYLER, EDWARD. The nicotinic acid, riboflavin, D-amino acid oxidase, and arginase levels of the livers of rats on a protein-free diet 1371

BORSOOK, HENRY, DEASY, CLARA L., HAAGEN-SMIT, A. J., KEIGHLEY, GEOFFREY, and LOWY, PETER H. The degradation of L-lysine in guinea pig liver homogenate: formation of α-aminoadipic acid 1383

BORSOOK, HENRY, DEASY, CLARA L., HAAGEN-SMIT, A. J., KEIGHLEY, GEOFFREY, and LOWY, PETER H. The degradation of α-aminoadipic acid in guinea pig liver homogenate 1395

HILLER, ALMA, PLAZIN, JOHN, and VAN SLYKE, DONALD D. A study of conditions for Kjeldahl determination of nitrogen in proteins. Description of methods with mercury as catalyst, and titrimetric and gasometric measurements of the ammonia formed 1401

HILLER, ALMA, GREIF, ROGER L., and BECKMAN, WILLIAM W. Determination of protein in urine by the biuret method 1421

HILLER, ALMA, PLAZIN, JOHN, and VAN SLYKE, DONALD D. Substitutes for saponin in the determination of oxygen and carbon monoxide of blood 1431

CHINARD, FRANCIS P. Interactions of quaternary ammonium compounds and proteins. A simple method for the rapid estimation of urinary protein concentrations with alkyldimethylbenzylammonium compounds 1439

CHINARD, FRANCIS P. Use of the hypobromite reaction for the estimation of ammonia plus urea nitrogen in urines containing large amounts of protein; the reaction of alkaline hypobromite with proteins........................ 1449

Letters to the Editors

SKEGGS, HELEN R., HUFF, JESSE W., WRIGHT, LEMUEL D., and BOSSHARDT, DAVID K. The use of *Lactobacillus leichmannii* in the microbiological assay of the "animal protein factor"........................ 1459

HEIDELBERGER, CHARLES, ABRAHAM, EDWARD P., and LEPKOVSKY, SAMUEL. Concerning the mechanism of the mammalian conversion of tryptophan into nicotinic acid........................ 1461

SHORB, MARY S., and BRIGGS, G. M. The effect of dissociation in *Lactobacillus lactis* cultures on the requirement for vitamin B_{12}........................ 1463

HOFFMANN, C. E., STOKSTAD, E. L. R., FRANKLIN, A. L., and JUKES, THOMAS H. Response of *Lactobacillus leichmannii* 313 to the antipernicious anemia factor........................ 1465

MATTSON, FRED H. The site of conversion of carotene to vitamin A............ 1467

GEYER, ROBERT P., CHIPMAN, JUNE, and STARE, F. J. Oxidation *in vivo* of emulsified radioactive trilaurin administered intravenously............... 1469

BENDICH, AARON, and BROWN, GEORGE BOSWORTH. 2,6-Diaminopurine, a precursor of nucleic acid guanine........................ 1471

HUGHES, D. E. Glutamine and the growth of *Staphylococcus aureus*............ 1473

KORNBERG, ARTHUR. The participation of inorganic pyrophosphate in the reversible enzymatic synthesis of diphosphopyridine nucleotide............. 1475

LILLIE, ROBERT J., DENTON, CHARLES A., and BIRD, H. R. Relation of vitamin B_{12} to the growth factor present in cow manure........................ 1477

INDEX TO VOLUME 176........................ 1479

THE DEPENDENCE OF THE SPECIFIC ACTIVITY OF UREASE UPON THE APPARENT ABSOLUTE ENZYME CONCENTRATION

BY JACK PETERSON,* KENT M. HARMON,† AND CARL NIEMANN

(*From the Gates and Crellin Laboratories of Chemistry,‡ California Institute of Technology, Pasadena*)

(Received for publication, May 22, 1948)

If the activity of an enzyme preparation is determined under conditions in which a further increase in substrate concentration is without demonstrable effect, all other factors being held constant, it is ordinarily assumed that the specific activity of the enzyme, expressed in terms of arbitrary units per unit weight of the enzyme, is independent of the absolute enzyme concentration (1, 2). However, with urease solutions stabilized with hydrogen sulfide or cysteine (1) we have observed that the specific activity of a given urease preparation, when determined under the above conditions, increases with decreasing apparent enzyme concentration over a wide range of concentrations and that this increase in specific activity proceeds with a measurable velocity at temperatures above 15°. This phenomenon was observed with crude urease preparations, such as jack bean meal, and with two, three, and seven times recrystallized urease. Since little or no difference was observed in the behavior of three and seven times recrystallized urease, the data presented in this paper are limited to those obtained with thrice recrystallized preparations. Urease activity was determined by a modification of the procedure described by Van Slyke and Cullen (3). The precision of the modified procedure was ±2 to 3 per cent.

EXPERIMENTAL

Determination of Urease Activity

Reagents—The buffer solution, 0.1 M in phosphate adjusted to pH 7.0, used in all experiments was prepared from dipotassium hydrogen phosphate and potassium dihydrogen phosphate. The 1.0 M solution of urea was prepared daily in order to minimize the effects of bacterial contamination. The crystalline urease was prepared from jack bean meal by the method of Dounce (4), all operations subsequent to the initial extraction being carried out at 5°. Thrice recrystallized urease from 200 gm. of meal was dissolved in 3 to 5 ml. of water, 1 per cent saturated with hydrogen sulfide, and this stock solution kept at 5° prior to its use. Hydrogen sulfide solutions were prepared daily by appropriate dilution of a solution saturated at 0°. Redistilled water was used in all cases.

---

* Died January 29, 1948.

† National Research Council Predoctoral Fellow.

‡ Contribution No. 1177.

Procedure—Clean 18 × 150 mm. reaction tubes were charged with 2.0 ml. of buffer solution and 1.0 ml. of 1.0 M urea solution (or standard ammonium sulfate solution containing 100 γ of ammonia nitrogen per ml.) and placed in a constant temperature bath at either 25° or 15°. When thermal equilibrium had been attained, the enzyme solution was added (usually 1.00 or 0.79 ml.) and the time noted. After the desired time interval had elapsed (usually 2 minutes), 0.5 ml. of 1.0 M sulfuric acid was added to each of the tubes; the latter were shaken and placed in an ice bath. Exactly 10 ml. of 0.01 M sulfuric acid were placed in each 18 × 150 mm. absorption tube and the tubes fitted with rubber stoppers, each bearing a 4 mm. glass inlet tube and a capillary type critical orifice designed to permit an air flow of 300 to 400 ml. per minute. To each of the reaction tubes in the ice bath were added 3 to 4 gm. of anhydrous potassium carbonate, and the tubes were fitted with rubber stoppers, each bearing a 4 mm. inlet tube as well as a 4 mm. U-tube serving to connect the reaction tube with the absorption tube. Each reaction tube was connected to an absorption tube with a short length of rubber tubing and placed in a 55° bath, the inlet and outlet tubes connected to manifolds, and the aeration started. After 20 minutes each reaction tube was disconnected and shaken so as to wash down the sides of the tube with carbonate solution. After this process, the tubes were reconnected and allowed to aerate for a second period of 20 minutes, when the washing process was repeated. After a final 20 minutes aeration the absorption tubes were disconnected and placed in an ice bath. To the chilled contents of each of the absorption tubes was added 1.0 ml. of Nessler's reagent (5), the tubes were removed from the ice bath, and after 10 minutes the intensity of the color measured in a Klett colorimeter. With the apparatus at our disposal it was possible to run eleven determinations simultaneously and in every such series two or more determinations were blanks in which either the urea solution or the urease solution was replaced by an equal volume of water or standard ammonium sulfate solution. For precise results such blank determinations were found to be necessary. The conversion of colorimeter readings to micromoles of ammonia was based upon a series of determinations of the ammonia recoverable in the range of 10 to 200 γ of ammonia nitrogen from a standard solution of ammonium sulfate. The specific activity was calculated from the relation specific activity = $10x/ta$, where x = the number of micromoles of ammonia liberated, t = the time in minutes during which hydrolysis occurred, and a = the micrograms of protein nitrogen present in the solution.

Dilution Experiments

Procedure—Relatively concentrated solutions of thrice recrystallized urease in water 1 per cent saturated with hydrogen sulfide, *i.e.*, containing 300 to 1500 γ of protein nitrogen per ml., were diluted with solutions of hydrogen sulfide or cysteine previously adjusted to pH 7.2 to 7.5 by the addition of anhydrous potassium carbonate.[1] The specific activity of urease in these solutions was then determined as a function of time, zero time being taken as the time of mixing. In those experiments in which a stepwise dilution technique was employed the solutions were allowed to stand at 25° for 2 hours after the initial dilution. A 1:10:6000 stepwise dilution is defined as an initial 1:10 dilution which has subsequently been diluted to effect a final over-all dilution of 1:6000. The data obtained in these experiments are given in Tables I to III.

Determination of Michaelis Constants

Procedure—The kinetics of the hydrolysis of urea by thrice recrystallized urease were studied at 25° and pH 7.0. The activity of the urease solutions was determined as described previously and the rate of determinations were made at nine different urea concentrations; *i.e.*, 0.004, 0.005, 0.006, 0.007, 0.008, 0.010, 0.015, 0.025, and 0.250 M urea. The data so obtained were found to obey the Michaelis-Menten equation, $v = VS/(Km + S)$, where v = the rate of hydrolysis at a urea concentration S and V = the maximum or limiting rate of hydrolysis over the concentration range of substrate studied. In practice it was found convenient to follow the suggestion of Lineweaver and Burke (6) and to transform the original Michaelis equation into its linear form $1/v = Km/VS + 1/V$ for evaluation of the data. It should be noted that the concentration of urea corresponding to a given rate was always taken as the average urea concentration obtaining during the determination of that rate.

DISCUSSION

The dependence of the specific activity of urease upon the apparent enzyme concentration in systems containing hydrogen sulfide was first observed when relatively concentrated solutions of urease in water containing hydrogen sulfide were diluted approximately a thousand fold with the same solvent.[2] It was further observed[2] that in the absence of hydrogen sulfide rapid inactivation of the urease occurred and that the effect observed with solutions containing hydrogen sulfide could be obviated by the presence of silver ion. In earlier experiments it was not appreciated that urease is slowly inactivated at pH 4.5 to 5.5 in solutions containing hydrogen sulfide. However, it was found that this inactivation could be minimized or suppressed by maintaining the systems at approximately pH 7 at all times.

---

[1] The pH of water 1 to 10 per cent saturated with hydrogen sulfide was found to vary between 5.5 and 4.5.

[2] Unpublished experiments of the authors.

The data presented in Columns 2 and 3 of Table I are typical of those observed when relatively concentrated solutions of urease are diluted at 25° and pH 7 with water containing hydrogen sulfide and the specific activity of the urease in these solutions determined as a function of time, with zero time as the time of mixing. The initial values are not particularly accurate, since the actual determination of urease activity requires a minimum of 2 minutes if precise results are to be obtained. Nevertheless the data in Columns 2 and 3 of Table I clearly illustrate that the specific activity of the urease in these solutions increases with time.

Table I

*Dilution of Urease Solutions with Water 1 Per Cent Saturated with Hydrogen Sulfide at 25° and pH 7.2 to 7.5**

Time after dilution	Specific activity at 25°, pH 7.0†					
	1:6000 dilution		1:4:6000 dilution		1:10:6000 dilution	
	Experiment I	Experiment II	Experiment I	Experiment II	Experiment I	Experiment II
(1)	(2)	(3)	(4)	(5)	(6)	(7)
min.						
2	136	131	138‡	146‡	156	148
10	141	134‡	145	151	161	153
30	149	139	156‡	154	168	154
70	169	163	186	170‡	181	163
120	186	175	207	186	196	184
180	198	191‡	218		203‡	
240	210	207	228	204	210‡	204
300	220		228		217	

* The original urease solution contained 1365 γ of protein nitrogen per ml.

† Average of duplicate determinations, agreeing within ±2 per cent.

‡ Obtained from a smooth curve through other points of the series.

The increase in the specific activity of urease upon dilution noted above was observed when urease solutions containing 1365 γ of protein nitrogen per ml. were diluted to give solutions containing approximately 0.23 γ of protein nitrogen per ml. In order to determine whether this effect could be observed with dilutions of lower order dilutions of 1:4 and 1:10 were employed; these solutions were allowed to stand sufficiently long to attain equilibrium, and then diluted uniformly to the point at which it was possible to determine the specific activity of the urease contained therein reasonably accurately. As before, the specific activity of the urease in the final solutions was determined as a function of time (Table I, Columns 4 to 7). It will be noted that in every case the specific activity increased with time and reached, within experimental error, the same maximum limiting value. Further, it is clear from these data that the initial 1:4 and 1:10 dilutions were effective in diminishing the percentage change in specific activity observable upon the final 1:6000 dilution, and one may conclude not only that dilutions of low order were attended by changes in specific activity, but also that at equilibrium and with relatively high concentrations of urease the specific activity of urease is a function of the enzyme concentration if the latter is taken as being equivalent to the amount of protein nitrogen present in solution.

TABLE II

*Dilution of Urease Solutions with Water 1 Per Cent Saturated with Hydrogen Sulfide at 15° and pH 7.3**

Time after dilution	Specific activity at 15°, pH 7.0, after 1:4000 dilution	
	Experiment I	Experiment II
min.		
2	88	83
15	88	
45	86	88
60	91	93

* The original urease solution contained 1365 γ of protein nitrogen per ml.

An attempt was made to estimate the magnitude of the temperature coefficient of the above reaction by measuring the change in the specific activity of urease brought about by a 1:4000 dilution of a relatively concentrated urease solution at 15° and 25° and at pH 7.3. Unfortunately the data obtained in these experiments (Table II) did not justify the calculation of a value for the temperature coefficient. However, they do provide evidence that the reaction is dependent on temperature.

The above observations on the behavior of urease in solutions containing hydrogen sulfide suggested the desirability of investigating the effects observable with another so called stabilizing agent. In Table III are presented data which were obtained in preliminary experiments in which cysteine was used instead of hydrogen sulfide. While these data are not as extensive as those available for hydrogen sulfide, it is clear that in both systems similar, but not necessarily identical, reactions are operative.

The Michaelis constant (1, 2, 7) of an enzyme is often taken as a characteristic property of the enzyme, though it is recognized (7) that the constant may be dependent upon temperature and pH.[3] Using crude preparations, Van Slyke and Cullen (3) and Ambros and Münch (8) obtained data which give values of 0.011 M urea at 20° and pH 7 and 0.0082 M urea at 50° and pH 7.6 for the Michaelis constant of urease. With urease solutions containing 0.065 to 0.095 γ of protein nitrogen per ml., which had been prepared by dilution with water 1 per cent saturated with hydrogen sulfide and allowed to stand at 25° for 3 to 4 hours, values for the Michaelis constant of five different urease preparations, obtained from two different lots of jack bean meal, were found to be 0.0098, 0.0116, 0.0098, 0.0112, and 0.0103 M urea at 25° and pH 7.0 respectively. The variation in the Michaelis constant noted above, *i.e.* 0.0107 ± 0.0009, was somewhat greater than that observed, *i.e.* 0.0106 ± 0.0003, when the constant of a given urease preparation was redetermined at intervals over a period of several months.

TABLE III

*Dilution of Urease Solutions with Solutions of Cysteine at 25° and pH 7.0**

Time after dilution	Specific activity at 25°, pH 7.0†			
	0.01 M cysteine	0.002 M cysteine		
	1:5000 dilution	1:5000 dilution	1:4:5000 dilution	1:10:5000 dilution
min.				
2	119	137	123	98
10	123	141	129	103
30	134	150	136	109
70	154	169	152	121
120	179	183	166	130
180	188	190	171	132‡
240	198	193	175	132
360	198	176	189	150

* The original urease solution contained 1365 γ of protein nitrogen per ml.

† Average of duplicate determinations, agreeing within ±2 per cent.

‡ Obtained from a smooth curve through other points of the series.

The fact that a substantial increase in the specific activity of urease is observed when a relatively concentrated solution of urease in water containing hydrogen sulfide or cysteine is diluted with the same solvent appears to preclude the possibility that the effect observed is simply an activation of urease by hydrogen sulfide or cysteine (9). An alternative explanation may be that the urease molecule dissociates into smaller units upon dilution and that this process is accompanied by an increase in the number of reactive sites, the hydrogen sulfide or cysteine merely serving to prevent inactivation. A second explanation may be that the crystalline urease preparations are contaminated with a naturally occurring inhibitor, which is not removed by the repeated recrystallization of urease from relatively concentrated solutions, and that the urease-inhibitor complex dissociates in dilute solutions, the degree of dissociation being a function, within limits, of the degree of dilution. Although there are insufficient data to determine whether all of the above hypotheses are operative or whether any one should be completely excluded, it is clear that the observed effect must be taken into account if studies on urease action are to be properly evaluated.

---

[3] Ionic strength and the nature of the buffer may also be important variables.

SUMMARY

It has been observed that the specific activity of urease in solutions containing hydrogen sulfide, or cysteine, and expressed in terms of arbitrary units of urease activity per unit weight of enzyme taken as protein nitrogen is dependent, within limits, upon the apparent enzyme concentration. The Michaelis constants of several urease preparations have been determined at 25° and pH 7.0 under conditions minimizing the above phenomenon.

BIBLIOGRAPHY

1. Sumner, J. B., and Somers, G. F., Chemistry and methods of enzymes, New York, 2nd edition (1947).
2. Van Slyke, D. D., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **2**, 33 (1942).
3. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, **19**, 211 (1914).
4. Dounce, A. L., *J. Biol. Chem.*, **140**, 307 (1941).
5. Bock, J. C., and Benedict, S. R., *J. Biol. Chem.*, **20**, 47 (1915).
6. Lineweaver, H., and Burke, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
7. Haldane, J. B. S., Enzymes, Monographs on biochemistry, London (1930).
8. Ambros, O., and Münch, H., *Z. physiol. Chem.*, **187**, 252 (1930).
9. Hellerman, L., Chinard, F. P., and Deitz, V. R., *J. Biol. Chem.*, **147**, 443 (1943).

STUDIES ON DIPEPTIDASES

II. SOME PROPERTIES OF THE GLYCYL-L-LEUCINE DIPEPTIDASES OF ANIMAL TISSUES*

By EMIL L. SMITH

(*From the Laboratory for the Study of Hereditary and Metabolic Disorders, and the Departments of Biological Chemistry and Medicine, University of Utah School of Medicine, Salt Lake City*)

(Received for publication, May 22, 1948)

One of the main problems connected with the study of peptidase activities concerns the individuality and specificity of the enzymes which are responsible for the hydrolysis of the many peptides which can be split by animal tissues. Thus far, only a few exopeptidases have been extensively studied, and the problem of classification of these activities demands considerable investigation prior to the actual isolation of these enzymes. The apparent instability of most of the enzymes which act on simple peptides has rendered the task of purification quite difficult. However, since it has become clear that many of the peptidases require the presence of heavy metals for full activity, the position is now somewhat better for establishing the conditions for the study of these enzymes.

The present investigation deals with the enzymes which are responsible for the hydrolysis of glycyl-L-leucine (GL) as these are found in hog intestinal mucosa, human uterus, and in rat and rabbit muscle. These enzymes all exhibit the specificity characteristics of dipeptidases, since the peptide derivatives which do not contain both free amino and free carboxyl groups are resistant to hydrolysis. Like the glycylglycine dipeptidase discussed earlier (2), the activity towards GL does not appear to parallel the hydrolysis of any other peptide which has been studied. The GL-splitting enzymes are, therefore, tentatively classified as specific glycyl-L-leucine dipeptidases.

Effect of Metal Ions

It has been found that the GL-splitting enzymes of different tissues may be quite specific in the type of metal which is required for activation. For example, with extracts of hog intestinal mucosa the hydrolysis of GL is activated by Mn^{++}, while the enzyme of human uterus is specifically ac-

---

* This investigation was supported by a grant from the United States Public Health Service. A report on a part of this work was presented at a meeting of the American Society of Biological Chemists at Atlantic City (March, 1948) (1). For the first paper in this series, see (2).

tivated by Zn++. A complicating factor is that all of these enzymes are inhibited by Ca++. Crude extracts of the tissues seldom exhibit their maximal activity, except in the presence of phosphate. The behavior of the enzymes in the presence of divalent metal ions will be discussed separately for the different tissues.

Human Uterus—Fig. 1 shows some of the data obtained with an aqueous extract of an acetone powder prepared from a filtered water extract of human uterus (3). In the presence of both Zn++ and phosphate, a maximal rate of splitting is found, and the reaction proceeds with the kinetics of a first order reaction. In the absence of phosphate (no buffer added), the hydrolysis is inhibited by Ca++ and, more surprisingly, by Zn++ also. Addition of phosphate alone gives a gradually increasing rate as compared to the control. Simultaneous addition of phosphate, Ca++, and Zn++ gradually reverses the inhibitory effect of Ca++.

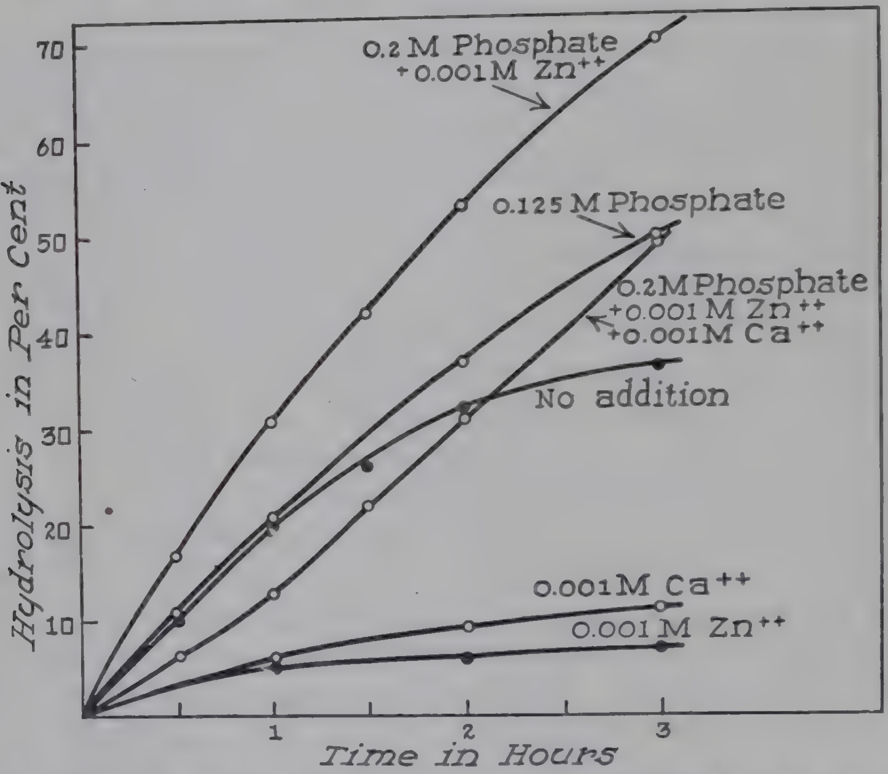


FIG. 1. Effect of metal ions and phosphate on the hydrolysis of glycyl-L-leucine by an enzyme preparation from human uterus. The experiments were performed at 40° at pH 7.8 to 8.0 at an enzyme concentration of 0.0102 mg. of protein N per cc.

While phosphate is fairly specific in allowing the maximal activity of the enzyme to be manifest, citrate is partially effective. The lower three experiments in Fig. 2 were conducted in the presence of 0.02 M veronal buffer. It is apparent, however, that highest activity is obtained only with the combination of Zn++ and phosphate, and that citrate is not completely effective in reversing the inhibitory effect which Zn^{++} has in the absence of phosphate. The GL dipeptidase of uterus is not activated by other metal ions which have been tested. In fact, inhibition is produced by Mg^{++} or Co^{++} (Table I).

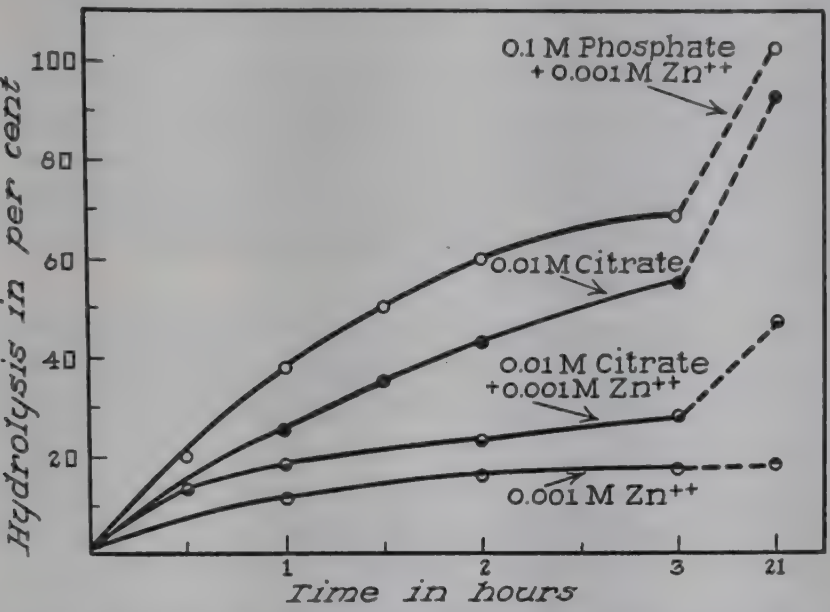


FIG. 2. Effect of citrate on the hydrolysis of glycyl-L-leucine by an enzyme from human uterus. The experiments were performed at 40° at pH 7.8 to 8.0 at an enzyme concentration of 0.0102 mg. of protein N per cc. The three experiments at the bottom of the graph were carried out in the presence of 0.02 M veronal buffer.

TABLE I

Effect of Metals on Hydrolysis of Glycyl-L-leucine

The experiments were performed at 40° and at pH 7.7 to 7.8. The metals were present at a concentration of 0.001 M. The preparations of hog intestinal mucosa and rat muscle were filtered aqueous extracts. The preparations from rabbit muscle and human uterus were made from acetone powders obtained from the water extracts of these tissues.

Tissue	Buffer	Protein N per cc. test solution	Time	Hydrolysis, per cent					
				No metal	Mn^{++}	Mg^{++}	Zn^{++}	Co^{++}	Fe^{++}
		mg.	*hrs.*						
Hog intestinal mucosa..	0.02 M veronal	0.52	0.5	76	39	45	32	20	41
			1	106	49	62	46	37	71
Rat muscle.............	0.02 “ “	0.47	1	69	40	67	21	41	72
	0.1 “ phosphate		1	72	51	79	83	51	63
Rabbit muscle..........	0.02 “ veronal	0.43	5	22	55	24	16	51	
			24	69	103	74	40	103	
Human uterus...........	0.1 “ phosphate	0.0069	2	31	19	27	45	14	

It should be noted that, in a few instances, Zn^{++} activated the enzyme even in the presence of veronal buffer (3). However, for completely reproducible results and for maximal activities, it has been found desirable to use phosphate buffer.

Rat Muscle—Crude aqueous extracts of rat muscle contain a GL dipeptidase which is very similar to that of human uterus. In veronal buffer, the enzyme is inhibited by 0.001 M Mn^{++}, Zn^{++}, Co^{++}, and Ca^{++}. Mg^{++} and Fe^{++} have no effect. In the presence of 0.1 M phosphate buffer, the enzyme is strongly activated by Zn^{++}, slightly activated by Mg^{++}, and is inhibited by Mn^{++} and Co^{++} (Table I).

Rabbit Muscle—Aqueous extracts of fresh muscle or of an acetone-dried powder show an Mn^{++} or Co^{++} activation of GL hydrolysis in the presence of veronal or phosphate. Other ions are inhibitory (Zn^{++}), or show no effect whatsoever (Mg^{++}).

Hog Intestinal Mucosa—A centrifuged aqueous extract of the mucosa displays no effect of activation, and the hydrolysis of GL is inhibited by 0.001 M Mn^{++}, Co^{++}, Mg^{++}, Zn^{++}, and Fe^{++} (Table I). A stable preparation may be obtained from the crude extract by collecting the precipitate which forms between 0.4 and 0.8 saturation with ammonium sulfate. The dialyzed and filtered solution shows a negligible activity in the absence of metal ions, but is strongly and specifically activated by Mn^{++}. This Preparation A was used for subsequent studies of kinetics and activation behavior.

Kinetics

It is evident from Table II that Preparation A of hog intestinal mucosa is strongly activated by Mn^{++}. If the enzyme is not preincubated with Mn^{++}, a considerable lag period is found during which the rate of hydrolysis of GL gradually increases. Thus, the reaction between Mn^{++} and protein to form an active enzyme is a time reaction like that previously described for other peptidases (3–5). After incubation of the protein with Mn^{++} for 3 hours, the hydrolysis of GL proceeds with the kinetics of a first order reaction.

When activation is produced by the specific metal with a crude tissue extract, no lag period is shown in the hydrolysis of the substrate, and the hydrolysis of GL proceeds as a first order reaction. For the different extracts, the velocity constants K are proportional to the enzyme concentration. Table III shows that with the uterine extract the proteolytic coefficient C is constant over a wide range in the presence of Zn^{++} and phosphate buffer. It should be noted that, for these experiments, satisfactory kinetic measurements can be made with as little as 0.00346 mg. of protein N per cc. of test solution. It is evident that the GL dipeptidase of uterine tissue is extremely active.

The experiments with the extract of rat muscle show satisfactory first order kinetics in the presence of Zn^{++} and phosphate. It is apparent from Table III that even in the presence of Mn^{++} and veronal, where the full activity of the enzyme is not manifest, K is proportional to the enzyme concentration.

The activation of hog mucosal Preparation A was studied at different Mn^{++} concentrations. A constant amount of enzyme (0.514 mg. of protein per cc.) was incubated with Mn^{++} for 3 hours and an aliquot was then added to the buffered substrate solution. The first order velocity constants are shown as a function of log Mn^{++} concentration in Fig. 3. The highest Mn^{++} concentration increases the rate of hydrolysis about 27-fold. The course of the activation is that of the typical mass law form previously described for other peptidases (2, 6). The apparent dissociation constant of the metal-protein complex is 1.8×10^{-4}. This value must be taken only as an approximation, since the experiments were performed in the presence of phosphate which may bind some of the metal. The true concentration of free Mn^{++} may be lower than that of the total Mn^{++} present in the test solutions.

TABLE II

Hydrolysis of Glycyl-L-leucine by Hog Intestinal Mucosa

The experiments were performed at 40° in 0.1 M phosphate buffer at pH 7.8. C is the proteolytic coefficient defined as the first order velocity constant K at enzyme concentration E, expressed in mg. of protein N per cc. where $C = K/E$. The enzyme was Preparation A.

Conditions	Enzyme concentration in test solution	Time	Hydrolysis	C
	mg. protein N per cc.	*hrs.*	*per cent*	
No metal added	0.103	3	1	
Contained 0.001 M Mn^{++}; no prior incubation	0.103	0.5	1	
		1	19	
		1.5	29	
		2	48	
Contained 0.003 M Mn^{++} in test solution after prior incubation for 3 hrs. of 0.02 M $MnCl_2$ with enzyme	0.072	0.5	17	0.036
		1	32	0.038
		1.5	38	0.032
		2	51	0.035
		2.5	58	0.035
		3	64	0.035

Specificity

Although the hydrolysis of dipeptides has frequently been ascribed to dipeptidases, evidence has seldom been presented in support of this view.

TABLE III

Effect of Enzyme Concentration on Hydrolysis of Glycyl-L-leucine

The measurements were performed with a crude filtered aqueous extract of rat muscle, with an aqueous extract of an acetone-dried powder of human uterus, and with a crude aqueous extract of hog intestinal mucosa. The tests with uterine extract were made at 40° in the presence of 0.001 M Zn^{++} and 0.15 M phosphate buffer at pH 7.8. The test solutions with the muscle extract contained 0.001 M Mn^{++} and veronal buffer. The experiments with the crude intestinal extract contained 0.04 M veronal and no added metal ion. The proteolytic coefficient $C = K/E$, where K is the first order velocity constant for the enzyme concentration E given in mg. of protein N per cc. of test solution.

Tissue extract	Enzyme concentration	Time	Hydrolysis	$K \times 10^{-3}$	C average
	mg. protein N per cc.	*hrs.*	*per cent*		
Human uterus	0.00346			1.04	0.30
	0.00692			1.98	0.29
	0.0102			3.04	0.30
	0.0138			4.0	0.29
				4.4	0.32
	0.0277			8.3	0.30
	0.0415			12.6	0.30
				11.9	0.29
	0.0692			19	0.27
				20	0.29
Rat muscle	0.109	7	28	0.34	
		24	73	0.39	0.0034
	0.163	3	18	0.46	
		5	33	0.57	
		6	39	0.60	
		7	46	0.64	0.0035
	0.218	2	18	0.73	
		4.5	35	0.70	
		5.5	43	0.75	
		7	54	0.81	0.0034
	0.272	2	22	0.89	
		3	33	0.95	
		4.5	46	0.97	
		5.5	54	0.99	0.0035
	0.408	1.5	29	1.67	
		2	36	1.59	
		2.5	42	1.55	
		3	47	1.55	
		4	57	1.55	0.0039
Hog intestinal mucosa	0.104			2.5	0.024
	0.26			6.4	0.025
	0.52			12.0	0.023

Maschmann (7) has suggested that the hydrolysis of certain dipeptides, including GL, is performed by substrate-specific enzymes. However, tests must be performed with appropriately substituted derivatives in order to determine the specificity of the enzyme involved in each instance. Thus, while the hydrolysis of glycylglycine is apparently due to a specific dipeptidase (2), the hydrolysis of L-leucylglycine is performed by an enzyme which has the specificity of an aminopeptidase (5, 8).

In Table IV are given some data on the specificity of the extracts of human uterus and of rat muscle. The uterine extracts show no splitting of carbobenzoxyglycyl-L-leucine, indicating that a carboxypeptidase is not involved and that a free amino group is essential. The slow hydrolysis of carbobenzoxyglycyl-L-leucinamide is not activated by Zn^{++} and requires at least 10 times the amount of enzyme necessary for hydrolysis of the free peptide. This hydrolysis must therefore be due to an endopeptidase of a different character.

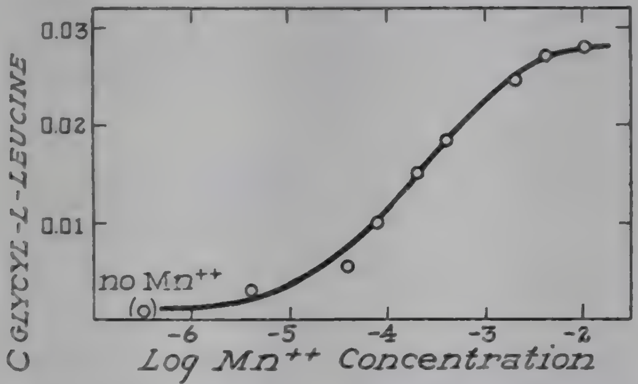


FIG. 3. Effect of concentration of Mn^{++} (in moles per liter) on the proteolytic coefficient for hydrolysis of glycyl-L-leucine by Preparation A of hog intestinal mucosa. For each experimental point, the enzyme (0.514 mg. of protein N per cc.) was incubated with Mn^{++} for 3 hours at 40° in the presence of 0.3 M phosphate buffer at pH 7.7. An aliquot was then added to the substrate solution buffered with 0.1 M phosphate at pH 7.8. The concentration of enzyme in each test solution was one-fifth of the incubation value. Correspondingly, the plotted Mn^{++} values are those for the test solutions; the incubation mixtures contained five times as much. Each plotted point is the average of two independent kinetic runs made on successive days.

With the uterine enzyme at a concentration of 0.082 mg. per cc., the hydrolysis of GL is nearly complete in 1 hour, while the hydrolysis of glycyl-L-leucinamide (GLA) proceeds quite slowly. With a 5-fold increase in enzyme concentration, it was noted that the initial hydrolysis of GLA is slightly inhibited by Zn^{++}, but is strongly activated by Mn^{++}. Moreover, in the presence of Mn^{++} both of the sensitive peptide bonds are hydrolyzed. It has recently been found that a highly purified preparation of leucine aminopeptidase rapidly hydrolyzes the amide bond of GLA to form GL and ammonia.[1] Since the aminopeptidase which is activated by Mn^{++} is present in all crude tissue extracts, it introduces a complicating factor.

---

[1] Smith, E. L., and Slonim, N. B., to be published.

It appears that the initial hydrolysis of GLA must take place at the amide bond by the action of the aminopeptidase; this is followed by the scission of the formed GL by the dipeptidase.

$$\begin{tikzcd}
\text{Glycyl-L-leucinamide} \arrow[r, "\text{Leucine aminopeptidase}", "\text{Mn}^{++}"'] & \text{Glycyl-L-leucine} + \text{NH}_3 \arrow[d, "\text{Zn}^{++}"', "\begin{array}{c}\textbf{Glycyl-L-leucine}\\ \textbf{dipeptidase}\end{array}"] \\
 & \text{Glycine} + \text{leucine}
\end{tikzcd}$$

TABLE IV

Specificity of Glycyl-L-leucine Dipeptidase

The experiments were performed at 40° in 0.1 M phosphate buffer at pH 7.8 with a substrate concentration of 0.05 M. Carbobenzoxyglycyl-L-leucinamide was only partially in solution. Hydrolysis is expressed as 100 per cent for the complete splitting of one peptide bond.

Tissue	Substrate	Enzyme concentration	Metal	Time	Hydrolysis
		mg. protein N per cc.		hrs.	per cent
Human uterus	Glycyl-L-leucine	0.082	0.001 M Zn++	1	91
	Carbobenzoxyglycyl-L-leucine	0.82	0.001 " "	22	4
	Carbobenzoxyglycyl-L-leucinamide	0.82	0.001 " "	5	28
				22	57
	Glycyl-L-leucinamide	0.082	0.001 " "	3	3
				20	16
	"	0.41	0.001 " "	1	9
				3	16
				20	75
	"	0.41	None	1	4
				3	13
				20	103*
	"	0.41	0.001 M Mn++	1	20
				3	95
				20	165*
	L-Leucinamide	0.205	0.001 " "	3	88
Rat muscle	Glycyl-L-leucine	0.24	0.001 " Zn++	3	84
			0.001 " Mn++	3	49
	Carbobenzoxyglycyl-L-leucine	0.95	0.001 " Zn++	23	3
	Carbobenzoxyglycyl-L-leucinamide	0.95	0.001 " "	23	10
			None	23	27
	Glycyl-L-leucinamide	0.24	0.001 M Zn++	3	16
				20	44
	"	0.24	0.001 " Mn++	3	31
				20	133*

* 100 per cent for the splitting of one peptide bond.

If the initial hydrolysis were due to the dipeptidase, Zn^{++} should exert a strong activating effect. Since it has not yet been possible to remove the aminopeptidase from these preparations, the question must remain open whether the enzyme which splits GL has a very slow action on GLA. Nevertheless, the GL-splitting enzyme does appear to have the specificity of a dipeptidase, since its action on the substituted compounds must be very slight indeed.

The crude extract of rat muscle shows a specificity similar to that of the uterine extract (Table IV). Here also, the differential effects of Zn^{++} and Mn^{++} on the splitting of GLA can be used to distinguish between the action of the Mn^{++}-activated leucine aminopeptidase present in this tissue (3) and the Zn^{++}-activated dipeptidase which acts on GL. With this extract, the endopeptidase action on carbobenzoxyglycyl-L-leucinamide is inhibited by Zn^{++}.

With extracts of hog intestinal mucosa and rabbit muscle, it is not possible to utilize the two metals in differentiating between the leucine aminopeptidase and the GL dipeptidase, since both of the enzymes from these tissues are activated by Mn^{++}. Hence, the consecutive splitting of both peptide bonds of GLA proceeds with great rapidity in the presence of Mn^{++}. Nevertheless, since the leucine aminopeptidase of hog mucosa has been obtained substantially free of the GL dipeptidase,[1] it appears legitimate to ascribe to the aminopeptidase all of the activity of the crude extract towards GLA, particularly since the purified enzyme shows this action to approximately the same relative extent as does the crude preparation.

DISCUSSION

The demonstration that the hydrolysis of GL is due to a dipeptidase provides the second instance in which it has been shown that the splitting of a dipeptide is due to a specific enzyme. The hydrolysis of glycylglycine has also recently been indicated to be due to a specific dipeptidase (2). Although the use of substituted compounds has provided the evidence for classifying these hydrolytic enzymes as dipeptidases, it is premature to conclude that other dipeptides are not hydrolyzed by these enzymes. The fact that Zn^{++} is a specific activator for two of the enzymes which split GL immediately differentiates these from the many other enzymes which split simple dipeptides and which are not activated by this metal. However, Zn^{++} has recently been reported to be the metal involved in a dehydropeptidase (8). We have found that the hydrolysis of glycyl-L-tryptophan by preparations from human uterus is also activated by Zn^{++}. Nevertheless, it can be stated that the hydrolysis of GL and of glycyl-L-tryptophan is due to distinct enzymes, since the GL dipeptidase is destroyed to a much greater extent by heating to 50° for 10 minutes than is the enzyme which splits glycyl-L-tryptophan. Up to the present time, we have not been able to obtain any evidence that the dipeptidase which splits GL possesses any other type of action.

The assistance of Mrs. Toshiko Shimizu is gratefully acknowledged.

EXPERIMENTAL

Hydrolysis was measured by the titration of liberated carboxyl groups by the method of Grassmann and Heyde (9). The substrates were present at a concentration of 0.05 M. Hydrolysis is expressed as 100 per cent for the complete splitting of one peptide bond. The acetone-dried preparations of human uterus and of the rat muscle extract have already been described (3). The substrates prepared were as follows: glycyl-L-leucine (10) and carbobenzoxyglycyl-L-leucine (11). Carbobenzoxyglycyl-L-leucinamide and glycyl-L-leucinamide hydrochloride will be described later.[1]

SUMMARY

1. The enzymes of human uterus and rat muscle which hydrolyze glycyl-L-leucine (GL) are activated by Zn^{++}; the corresponding enzymes of rabbit muscle and hog intestinal mucosa are activated by Mn^{++}. Maximal activity in all cases is obtained with phosphate buffer apparently because of the binding of Ca^{++} which is an inhibitor of these enzymes.

2. With activating metal and phosphate buffer, the hydrolysis of GL proceeds with the kinetics of a first order reaction, and is proportional to the enzyme concentration over a wide range.

3. After precipitation and dialysis of the GL dipeptidase of hog intestinal mucosa, the activation by Mn^{++} is a time reaction. The activity of the enzyme at different Mn^{++} concentrations shows a typical mass law relationship with an apparent dissociation constant of 1.8×10^{-4}.

4. The GL-splitting enzymes possess the specificity of dipeptidases, since carbobenzoxyglycyl-L-leucine, carbobenzoxyglycyl-L-leucinamide, and glycyl-L-leucinamide are hydrolyzed either not at all or only very slowly by the dipeptidases.

5. Extracts of human uterus and rat muscle contain an endopeptidase which slowly splits carbobenzoxyglycyl-L-leucinamide. This hydrolysis is not activated by metal ions.

BIBLIOGRAPHY

1. Smith, E. L., *Federation Proc.*, **7,** 189 (1948).
2. Smith, E. L., *J. Biol. Chem.*, **173,** 571 (1948).
3. Smith, E. L., *J. Biol. Chem.*, **173,** 553 (1948).
4. Smith, E. L., and Bergmann, M., *J. Biol. Chem.*, **138,** 789 (1941).

5. Smith, E. L., and Bergmann, M., *J. Biol. Chem.*, **153,** 627 (1944).
6. Smith, E. L., *J. Biol. Chem.*, **163,** 15 (1946).
7. Maschmann, E., *Biochem. Z.*, **319,** 28 (1941).
8. Yudkin, W. H., and Fruton, J. S., *J. Biol. Chem.*, **170,** 421 (1947).
9. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183,** 32 (1929).
10. Fischer, E., and Steingroever, J., *Ann. Chem.*, **365,** 167 (1909).
11. Stahmann, M. A., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **164,** 753 (1946).

STUDIES ON DIPEPTIDASES

III. HYDROLYSIS OF METHYLATED PEPTIDES; THE RÔLE OF COBALT IN THE ACTION OF GLYCYLGLYCINE DIPEPTIDASE*

By EMIL L. SMITH

(*From the Laboratory for the Study of Hereditary and Metabolic Disorders, and the Departments of Biological Chemistry and Medicine, University of Utah School of Medicine, Salt Lake City*)

(Received for publication, May 22, 1948)

The recognition that the hydrolysis of the peptides, glycylglycine and glycyl-L-leucine, is due to specific dipeptidases (1–3) has led to a search for an explanation of why the responsible enzymes should require such extremely specific substrate configurations. While a complete description is obviously not yet possible, a partial answer seems to be in the rôle that certain metals play in these hydrolytic reactions. It was suggested that, in the case of glycylglycine dipeptidase, the function of Co^{++} is to act as a bridge forming a coordination compound involving the substrate, on the one hand, and the protein on the other (1). This hypothesis arose from the observation that at pH 7.6, where the enzyme is maximally active, Co^{++} coordinates much more readily with glycylglycine (GG) than it does with triglycine or glycine, as judged by the relative effect of these compounds on the absorption spectrum of Co^{++}. This suggestion has now been subjected to further test in several ways. First of all, it has been found that those substituted compounds which are not split by the enzyme react either weakly or not at all with Co^{++}. Secondly, an additional substrate for the GG dipeptidase has been found in sarcosylglycine (SG), and this compound coordinates readily with Co^{++}. From the closely parallel data of the specificity of the enzyme and the specificity of complex formation with Co^{++}, some evidence is available regarding the types of linkage involved.

The observations with Co^{++} are facilitated by the characteristic absorption bands which can easily be studied, unlike the coordination of other metals with peptides in which we have observed only a change in the ultraviolet end-absorption. On the other hand, certain difficulties appear in this work with Co^{++}; namely, the reversible oxygen binding and subsequent irreversible slow oxidation which may occur with some of these chelate compounds, as beautifully demonstrated by Burk and his collaborators (4). Nevertheless, that other effects may be found in the absence of the enzyme should not detract from the primary matter of whether or not coordination takes place.

---

* This work was aided by a grant from the United States Public Health Service.

Specificity of Glycylglycine Dipeptidase

It is shown in Table I that two tissue extracts rich in this dipeptidase (1) readily split SG and that this hydrolysis is strongly accelerated by Co^{++} in the same degree as the hydrolysis of GG. Neither of these extracts has the slightest detectable effect on glycylsarcosine or N-dimethylglycylglycine; this is particularly striking with the uterine extract, where no splitting was observed in 21 hours with 40 times the amount of enzyme necessary for complete hydrolysis of GG in 3 hours. Although glycylsarcosine has previously been shown to be resistant to hydrolysis (5, 6), this has been reinvestigated because of the pronounced activation of GG dipeptidase by Co^{++}, which was unknown when the earlier work was performed.

TABLE I

Specificity of Glycylglycine Dipeptidase

The experiments were performed at 40° in 0.1 M phosphate buffer at pH 7.6 to 7.7.

Tissue	Substrate	Protein N per cc. test solution	Time	Hydrolysis	
				0.001 M Co^{++}	No Co^{++}
		mg.	*hrs.*	*per cent*	*per cent*
Rat muscle	Glycylglycine	0.20	3	93	18
	Sarcosylglycine	0.60	20	63	13
	N-Dimethylglycylglycine	0.80	20	−2	3
	Glycylsarcosine	0.80	20	0	1
Human uterus	Glycylglycine	0.02	3	104	6
	Sarcosylglycine	0.40	3	102	3
	N-Dimethylglycylglycine	0.80	21	1	2
	Glycylsarcosine	0.80	21	2	−2

It has already been demonstrated that the hydrolysis of GG follows the kinetics of a zero order reaction with extracts of human uterus or rat muscle (1); this is likewise true for the enzyme of hog intestinal mucosa (Table II). With all three extracts, the hydrolysis of SG also follows the zero order kinetics. K^0, the zero order velocity constant, is calculated as per cent hydrolysis per minute, and C^0 is K^0/E, where E is in mg. of protein N per cc. K^0_{SG} is proportional to the enzyme concentration, as shown in Table II for the uterine extract.

The GG dipeptidase from hog intestinal mucosa is relatively labile even on standing in the cold. After 5 days, C^0_{GG} decreased from 15.3 to 4.6, or 3.3 times. Similarly, C^0_{SG} decreased from 1.07 to 0.29 or 3.7 times. The parallel loss of activity for the two substrates is further evidence that the same enzyme is responsible for both actions. Similar results have also been obtained with the labile GG dipeptidase of rat muscle.

From the data in Table II, the proteolytic quotient Q, C^0_{GG}/C^0_{SG}, may be calculated for the three extracts. For rat muscle $Q = 11.7$, for intestinal mucosa $Q = 14.3$, and for uterus $Q = 4.8$. If the activities were completely homospecific, Q should be the same for all three extracts. With extracts

TABLE II

Kinetics of Hydrolysis of Sarcosylglycine

The tests were made at 40° in the presence of 0.001 M $CoCl_2$. The solutions were buffered at pH 7.6 to 7.8 with 0.02 M veronal (rat muscle) or 0.1 M phosphate (uterus and intestinal mucosa). The preparation from intestinal mucosa was a fresh aqueous extract of an acetone-dried powder; the aged extract had been allowed to stand at 5° for 5 days. K^0 is the zero order velocity constant expressed as per cent hydrolysis per minute. C^0, the zero order proteolytic coefficient, is K^0/E, where E is in mg. of protein N per cc.

Tissue	Substrate	Protein N per cc. test solution	Time	Hydrolysis	K^0	C^0
		mg.	*min.*	*per cent*		
Rat muscle	Glycylglycine	0.059			0.61	10.4
	Sarcosylglycine	0.108	180	17	0.094	
			240	23	0.096	
			300	29	0.097	0.89
Human uterus	Glycylglycine	0.041			0.69	16.8
		0.082			1.28	15.6
	Sarcosylglycine	0.082			0.31	3.8
		0.123			0.41	3.3
		0.205			0.62	3.0
		0.246			0.87	3.5
		0.41	15	19	1.27	
			30	38	1.27	
			45	56	1.24	
			60	75	1.25	3.1
Hog intestinal mucosa	Glycylglycine	0.0416	30	17	0.57	
			60	38	0.63	
			90	61	0.68	
			120	79	0.66	15.3
		0.0416	120	17*		
	Sarcosylglycine	0.208	90	19	0.21	
			150	36	0.24	
			180	40	0.22	
			300	65	0.22	1.07
			300	19*		
" " "	Glycylglycine	0.0416			0.19	4.6
(aged 5 days)	Sarcosylglycine	0.208			0.06	0.29

* These test solutions did not contain added Co^{++}.

of rat muscle and hog mucosa, consistent values of Q were always obtained (Table III). However, with some extracts of human uterus values of 10 to 12 were found. While the data were reasonably consistent for C^0_{GG}, the variation appears to be in the rate of hydrolysis of SG. From this, it would appear that the GG dipeptidases of different tissues are homospecific, but there is probably some additional factor in uterine extracts which is concerned in the hydrolysis of SG.

TABLE III

Proteolytic Quotients for Glycylglycine Dipeptidases

The experiments were performed at 40° and pH 7.6 to 7.8 with 0.02 M veronal or 0.1 M phosphate in the presence of 0.001 M Co^{++}. The proteolytic quotient = C^0_{GG}/C^0_{SG}.

Preparation	Buffer	C^0_{GG}	C^0_{SG}	Proteolytic quotient
1.* Human uterus	Phosphate	16.2	3.4	4.8
2. " "	"	15.3	3.1	4.9
3. " "	"	18.8	4.6	4.1
4. " "	Veronal	13.8	1.37	10.1
5. " "	Phosphate	18.8	1.61	11.7
6. " "	Veronal	12.2	1.07	11.4
1.* Rat muscle	"	10.4	0.98	11.7
2. " "	"	10.3	0.81	12.7
3. " "	Phosphate	4.5	0.46	9.8
1.* Hog intestinal mucosa	"	15.3	1.07	14.3
2. " " "	"	14.5	0.89	16.3
3. " " "	"	11.5	1.03	11.2

* These are the preparations used for the experiments in Table II.

Hydrolysis of Sarcosyl-L-leucine

Thus far, the only other specific dipeptidase action has been ascribed to the enzymes of various tissues which split glycyl-L-leucine (GL) (2, 3). It was of interest to ascertain whether these enzymes are capable of acting on the analogous methylated peptide, sarcosyl-L-leucine (SL). It was found that the Mn^{++}-activated GL dipeptidase of hog intestinal mucosa does hydrolyze SL. The splitting of SL is also activated by Mn^{++}, and shows a first order rate of hydrolysis only after the enzyme has been preincubated with Mn^{++} (Table IV). Similarly, the Zn^{++}-activated dipeptidase of human uterus hydrolyzes SL and this hydrolysis is activated by Zn^{++} also. This splitting of SL likewise follows the kinetics of a first order reaction. It should be noted that for the intestinal preparation Q for C_{GL}/C_{SL} is 17, while for the uterine extract Q is 74. Obviously, the two systems do not appear to be completely homospecific.

Coordination Compounds of Cobalt and Peptides

Measurements of absorption spectra were made in a 1 cm. quartz cell at room temperature with a Beckman ultraviolet spectrophotometer. A solution of the compound was adjusted to pH 7.6 to 7.8 with 0.1 M NaOH, and $CoCl_2$ was added. The final concentrations were 0.125 M for the compound and 0.01 M Co^{++}. The spectrum was measured after the solution was allowed to stand for 18 to 24 hours. Since no anaerobic precautions were taken, those compounds which coordinated with Co^{++} may have undergone some formation of the oxygen binding and oxidation complexes described by Burk *et al.* (4). However, the interest in this study was whether coordination took place at all, and not in the secondary fate of the coordination compound in the absence of the enzyme. It has already been demonstrated (1) that the product formed by the action of the enzyme on GG is glycine.

TABLE IV

Hydrolysis of Sarcosyl-L-leucine

The tests were performed at 40° and pH 7.7 in 0.1 M phosphate. The intestinal Preparation A was incubated at 40° for 3 hours with 0.02 M Mn^{++} before addition to the test solution. C^1 is the first order proteolytic coefficient.

Tissue	Substrate	Metal in test solution	Protein N per cc. test solution	Time	Hydrolysis	C^1
			mg.	*hrs.*	*per cent*	
Hog intestinal mucosa	Glycyl-L-leucine	0.002 M Mn^{++}	0.103			0.031
	Sarcosyl-L-leucine	0.007 " "	0.362			0.0018
		None	1.03			0.00015
Human uterus	Glycyl-L-leucine	0.001 M Zn^{++}	0.0197			0.31
	Sarcosyl-L-leucine	0.001 " "	0.59	0.5	16	0.0043
				1.0	31	0.0046
				1.5	40	0.0043
				2.0	46	0.0038
				2.5	56	0.0040
	"	None	0.59	1.0	18	0.0025
				2.0	30	0.0022
				2.5	35	0.0021

The following glycine derivatives which are not hydrolyzed by GG dipeptidase showed no change or only a very small increase in the intensity of the Co^{++} absorption spectrum: glycinamide, benzoylglycine, benzoylglycylglycine, carbobenzoxyglycylglycine, glycylsarcosine, and N-dimethylglycylglycine. The sparingly soluble benzoylglycinamide and carbobenzoxyglycylglycinamide did not show any reaction with Co^{++}. In addition, no coordination took place with β-alanylglycine or β-alanyl-β-alanine, but glycyl-β-alanine and glycyl-L-alanine did show slight evidence of coordination.

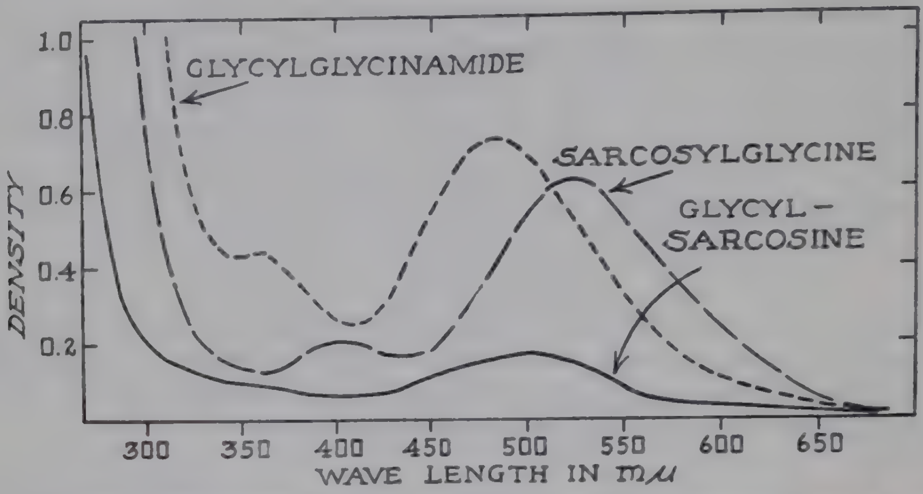


FIG. 1. Absorption spectra of sarcosylglycine, glycylsarcosine, and glycylglycinamide after standing with 0.01 M $CoCl_2$ for 24 hours.

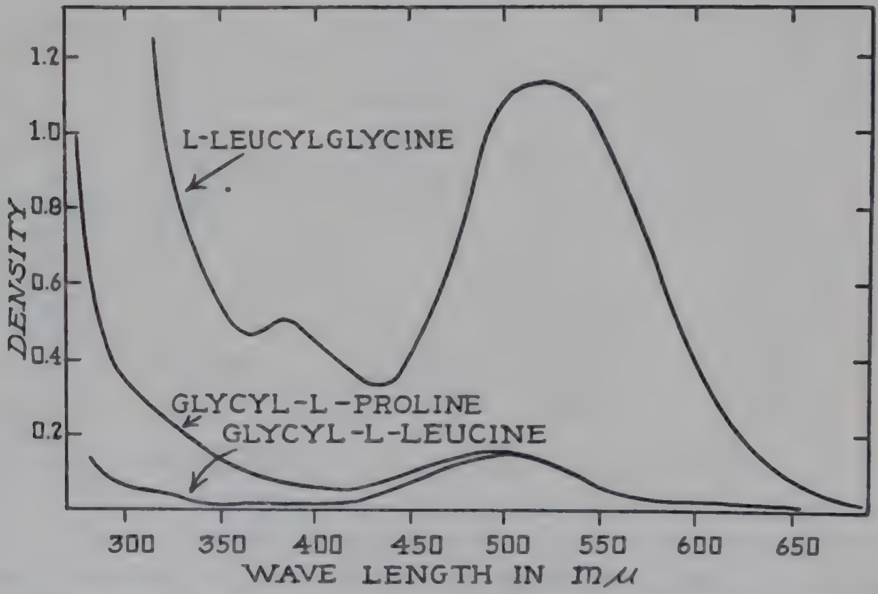


FIG. 2. Absorption spectra of L-leucylglycine, glycyl-L-leucine, and glycyl-L-proline after standing with 0.01 M $CoCl_2$ for 24 hours.

It has already been reported (1) that GG forms a compound which gives a strong intensification of the absorption bands of Co^{++}. This compound has its maximal absorption of 520 mμ. Sarcosylglycine forms a similar complex, and the maximal absorption in the visible is at the same wave-length (Fig. 1). However, the intensity of the band is somewhat less than that of the GG complex. Glycylglycinamide, which is resistant to the dipeptidase, also forms a coordination complex with Co^{++} (Fig. 1). However, while the height of the band is like that of SG rather than GG, the position of the maximum is at 480 mμ. The spectrum of the weak complex of Co^{++} with glycylsarcosine is also shown in Fig. 1. $CoCl_2$ (0.01 M) has an absorption maximum at 510 mμ with an optical density of 0.05.

It was found that L-leucylglycine coordinates much more strongly with Co^{++} than does glycyl-L-leucine (Fig. 2). Obviously, there is considerable specificity in the formation of these complexes, even with unsubstituted peptides. The peptide glycyl-L-proline, which does not possess a peptide hydrogen, shows only weak coordination with Co^{++}.

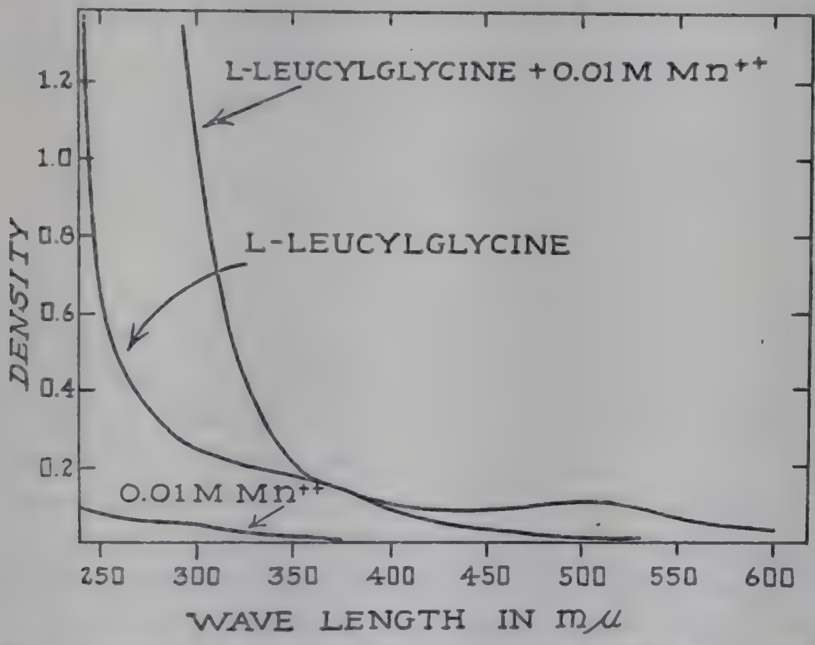


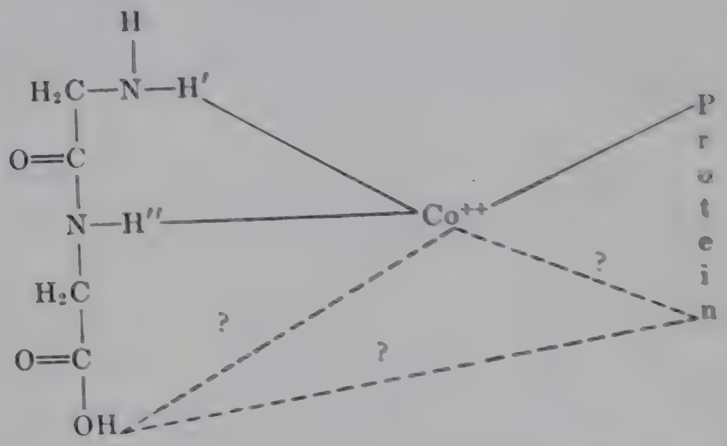
FIG. 3. The absorption spectrum of L-leucylglycine (0.125 M), which had been allowed to stand with 0.01 M $MnCl_2$ for 24 hours at room temperature. This is compared with the spectrum of the free peptide and 0.01 M $MnCl_2$.

Complex formation also occurs with other metals and peptides. Fig. 3 shows the change in the absorption spectrum which occurs with L-leucylglycine and Mn^{++} at pH 7.7. A similar but weaker increase in absorption may also be demonstrated with L-leucylglycine and Mg^{++}. This is of considerable interest, since the ubiquitous leucine aminopeptidase which splits this peptide is activated by Mn^{++} and Mg^{++}.

DISCUSSION

An attempt has been made to test the hypothesis that the rôle of metals in hydrolytic and other non-oxidative reactions is to act as a bridge in the formation of the enzyme-substrate complex. This has been investigated by the study of complex formation between Co^{++} and a series of compounds related to glycylglycine, since this peptide is hydrolyzed by a specific enzyme which requires Co^{++} for its activity. An obvious parallelism has been found between the compounds which form a Co^{++} complex and those which are split by the dipeptidase. This is summarized in Table V. The data are taken from this paper and an earlier one (1).

The data indicate to some extent the manner in which complex formation must take place.[1] Since sarcosylglycine forms a compound with Co^{++} and



is hydrolyzed by the dipeptidase, one free hydrogen (H′) is sufficient on the amino group. The nitrogen must be basic since the acylated compounds, carbobenzoxyglycylglycine and benzoylglycylglycine, do not coordinate and are not split. The inactivity of the strongly basic compound N-dimethylglycylglycine shows that basicity is not a sufficient requirement; one free hydrogen must be present on the terminal amino group.

It is well known that Co^{++} coordinates best with compounds like ethylenediamine, where chelate rings are possible. Therefore, the peptide hydrogen (H″) must be present for a chelate complex to be formed. The weak complex of glycylsarcosine with Co^{++} shows this to be the case, and this compound is completely resistant to the enzyme. The tendency of Co^{++} to form five-membered chelate rings is illustrated by the failure to observe any coordination with β-alanylglycine and β-alanyl-β-alanine; neither of these compounds is hydrolyzed by GG dipeptidase (7). However, the enzyme may have a slow action on glycyl-β-alanine (7) and there is some evidence of weak coordination of this compound with Co^{++}.

Glycylglycinamide is not hydrolyzed by the dipeptidase. However, it does form a chelate complex with Co^{++} but of a somewhat different character than glycylglycine as indicated by the shift of 40 mμ in the principal absorption band. Whether the free carboxyl group is necessary for combination with Co^{++} or with the protein is still undecided, although it would seem probable that Co^{++} forms a chelate complex with the protein also. Of the four coordination places which Co^{++} has available, two must be with the peptide, and at least one with the protein. Complex formation between metals and proteins is, of course, quite well known (8). The fact that for certain enzymes the activation by the metal is a slow reaction indicates that complex formation, and not a simple ionic reaction, is occurring. This was first described for leucine aminopeptidase (9, 10) and has since been found for arginase (11), phosphatase (12), prolidase (13), and glycyl-L-leucine dipeptidase (3). The rate of such reactions seems to vary quite widely, and to depend on many factors. The more highly purified enzymes seem to react more slowly than do many crude preparations. Thus far, we have been unable to detect any activation time of glycylglycine dipeptidase and Co^{++}; nor have we observed any increase in rate of splitting of GG by prior incubation of substrate and Co^{++}.

---

[1] In the absence of the enzyme, coordination probably involves 2 moles of the compound with 1 of Co^{++} for those substances in which formation of the chelate compound is possible. However, with molecules like glycine, 4 moles would coordinate with each Co^{++}.

TABLE V

Action of Glycylglycine Dipeptidase and Complex Formation with Co^{++}

The optical densities (1 cm. cell) were measured with a solution of the compound (0.125 M) and $CoCl_2$ (0.01 M) at pH 7.6 to 7.8 after 18 to 24 hours at room temperature.

Compound	Hydrolysis by dipeptidase	Optical density	Wave-length
			mμ
Glycylglycine	++++	2.0*	520
Sarcosylglycine	++	0.60	520
N-Dimethylglycylglycine	−	†	
Glycylsarcosine	−	0.17	500
Glycylglycinamide	−	0.73	480
Glycinamide	−	0.15	500
Glycine		0.16	500
Triglycine	−	0.49	500
Benzoylglycylglycine	−	0.06	510
β-Alanylglycine	−	†	
β-Alanyl-β-alanine	−	†	
Glycyl-β-alanine	±	0.17	500
Glycyl-L-alanine	−	0.28	505
0.01 M $CoCl_2$		0.06	510

* After 3 hours, the value was 0.52.

† Precipitation of gelatinous cobalt hydroxide.

It is apparent from this hypothesis that a part of the specificity of the enzyme must reside in the ability of the metal to undergo complex formation both with the substrate and with the protein. This would help to explain the extreme metal specificity shown by many enzymes, including the peptidases. However, this must be stated as a necessary condition for enzyme action, but is not a sufficient condition, since the major enzyme specificity must be due to the protein moiety.

Green, Herbert, and Subrahmanyan (14) have proposed that the rôle of the metal ion in carboxylase is to bind diphosphothiamine to the protein moiety of the enzyme. However, their data do not exclude the possibility that the linkage is in the order protein-diphosphothiamine-metal. With the peptidases, no other dissociable prosthetic group other than metal has yet been found, although leucine aminopeptidase and prolidase have now been extensively purified. Here the metal must form the link between the enzyme and the substrate.

Thus far, the hypothesis of the rôle of Co^{++} in the action of glycylglycine dipeptidase has fulfilled every test to which it has been subjected. It is likely that this proposal can be extended to other peptidases and additional types of enzymes. No other suggestion to explain why metals are required in hydrolytic reactions has been proposed, and it is difficult to conceive of any other function which these metals could have.

The assistance of Douglas M. Brown and Mrs. Toshiko Shimizu is gratefully acknowledged.

EXPERIMENTAL

Hydrolysis was measured on 0.2 cc. samples by the liberation of carboxyl groups by the method of Grassmann and Heyde (15). The substrates were used at 0.05 M in 2.5 cc. volumetric flasks. The rat muscle extract and the acetone-dried preparation of human uterus have already been described (1). For glycylglycine dipeptidase, a fresh aqueous extract of hog intestinal mucosa was used. For glycyl-L-leucine dipeptidase, the crude aqueous extract of mucosa was precipitated with ammonium sulfate and dialyzed (3).

Sarcosyl-L-leucine—6 gm. of chloroacetyl-L-leucine (16) were allowed to stand in a pressure bottle at room temperature for 2 days with 30 cc. of 35 per cent aqueous methylamine. The solution was diluted with water and concentrated to a thick syrup under reduced pressure repeatedly with ethanol. The gelatinous residue was dissolved in ethanol and precipitated with ether. Yield, 5.1 gm. The compound was recrystallized from ethanol as needles; m.p. 187°.

$C_9H_{18}O_3N_2$.	Calculated.	C 53.4, H 9.0, N 13.9
202.3	Found.	" 53.4, " 9.0, " 13.8

$[\alpha]_D^{21.6} = -30.4°$ (2.2% in water)

N-Dimethylglycylglycine Hydrochloride—5 gm. of chloroacetylglycine (17) were dissolved in 20 cc. of 25 per cent dimethylamine and allowed to stand at room temperature for 4 days. The solution was concentrated *in vacuo* repeatedly with ethanol. The thick syrup was taken up in hot butanol and filtered. On cooling the solution, 3.4 gm. of the crystalline product were obtained. In order to remove the dimethylamine hydrochloride, the substance was dissolved in water and crystallized as fine needles by the addition of ethanol. After one more recrystallization, the melting point was 186–187°.

$C_6H_{13}O_3N_2Cl$.	Calculated.	C 36.7, H 6.7, N 14.3
196.6	Found.	" 36.7 " 6.6, " 14.3

Sarcosylglycine—This was prepared as described by Levene, Simms, and Pfaltz (17).

Glycylsarcosine—This was synthesized by the procedure of Bergmann, Zervas, Schleich, and Leinert (6).

SUMMARY

1. Preparations of glycylglycine dipeptidase from human uterus, rat muscle, and hog intestinal mucosa hydrolyze sarcosylglycine. This hydrolysis, like that of glycylglycine, is strongly activated by Co^{++}, and follows the kinetics of a zero order reaction. The enzyme has no demonstrable action on glycylsarcosine or N-dimethylglycylglycine.

2. The glycyl-L-leucine dipeptidases of hog intestinal mucosa (Mn^{++} activation) and human uterus (Zn^{++} activation) hydrolyze sarcosyl-L-leucine. These splittings follow first order reaction kinetics.

3. Co^{++} coordinates readily with glycylglycine but not with most of the substituted derivatives of the dipeptide. In view of the parallelism between the ability of Co^{++} to coordinate and the ability of the enzyme to act, it is proposed that the rôle of metals in hydrolytic reactions is to act as a bridge in the formation of the enzyme-substrate complex.

BIBLIOGRAPHY

1. Smith, E. L., *J. Biol. Chem.*, **173**, 571 (1948).
2. Smith, E. L., *Federation Proc.*, **7**, 189 (1948).
3. Smith, E. L., *J. Biol. Chem.*, **176**, 9 (1948).
4. Burk, D., Fiala, S., Hearon, J., Hesselbach, M., Levy, H., and Schade, A. L., *Federation Proc.*, **7**, 148 (1948).
5. Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, **62**, 711 (1924–25).
6. Bergmann, M., Zervas, L., Schleich, H., and Leinert, F., *Z. physiol. Chem.*, **212**, 72 (1932).
7. Hanson, H. T., and Smith, E. L., *J. Biol. Chem.*, **175**, 833 (1948).

8. Schmidt, C. L. A., The chemistry of the amino acids and proteins, Springfield, 720 (1938).
9. Smith, E. L., and Bergmann, M., *J. Biol. Chem.*, **138**, 789 (1941); **153**, 627 (1944).
10. Smith, E. L., *J. Biol. Chem.*, **163**, 15 (1946).
11. Hunter, A., and Downs, C. E., *J. Biol. Chem.*, **155**, 173 (1944).
12. Roche, J., Thoai, N.-V., and Roger, M., *Arch. internat. physiol.*, **54**, 209 (1946).
13. Smith, E. L., *J. Biol. Chem.*, **173**, 553 (1948).
14. Green, D. E., Herbert, D., and Subrahmanyan, V., *J. Biol. Chem.*, **138**, 327 (1941).
15. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183**, 32 (1929).
16. Fischer, E., and Steingroever, J., *Ann. Chem.*, **365**, 167 (1909).
17. Levene, P. A., Simms, H. S., and Pfaltz, M. H., *J. Biol. Chem.*, **61**, 445 (1924).

THE SYNTHESIS OF S-BENZYLTHIOPYRUVIC ACID AND ITS CONVERSION TO N-ACETYL-S-BENZYL-L-CYSTEINE IN THE RAT; UNAVAILABILITY OF THIOPYRUVIC ACID TO RATS FOR GROWTH PURPOSES*

By JAKOB A. STEKOL†

(From the Lankenau Hospital Research Institute and The Institute for Cancer Research, Philadelphia)

(Received for publication, May 13, 1948)

Some time ago we demonstrated the acetylation of S-benzyl-L-cysteine in the dog, rabbit, rat, and man (1–3). Administration of S-benzyl-D-cysteine to rats (4, 5) or man (3) was followed by the excretion of some N-acetyl-S-benzyl-L-cysteine, thus indicating a partial inversion of the D derivative of cysteine to the optical antipode. Ample experimental evidence is available to show that oxidative deamination of D-amino acids occurs *in vivo* and *in vitro*. In an attempt to explain the inversion of S-benzyl-D-cysteine *in vivo*, it has been postulated that S-benzylthiopyruvic acid is formed *in vivo* as the intermediate (2–5). S-Benzyl-L-cysteine is readily deaminated oxidatively by rat kidney preparations (6), and a hydrazone of a keto acid has been isolated from rabbit urine following the administration of S-benzyl-L-cysteine, which was presumably the hydrazone of S-benzylthiopyruvic acid (7). Previous experiments have shown that the α-hydroxy analogues of S-benzyl-L- and S-benzyl-DL-cysteine are excreted unchanged by the rat, suggesting that the rat is unable to oxidize the hydroxy acid or to aminate it (8).

In the present study we prepared S-benzylthiopyruvic acid and administered it to adult rats. From the urine of these animals we isolated N-acetyl-S-benzyl-L-cysteine and identified it in a conventional manner. The demonstration of the asymmetric synthesis of L-cysteine derivative from the corresponding keto acid raised the question of a similar synthesis of L-cysteine from thiopyruvic acid *in vivo*. It will be recalled from earlier studies that D-cystine could not replace L-cystine in the diet of the rat for growth purposes (9), although D-cystine was shown to undergo ready oxidative deamination *in vitro* by rat kidney preparations (10). It thus appeared possible that D-cystine undergoes oxidative deamination *in vivo* also, but the resulting thiopyruvic acid is metabolized before the synthesis of L-cystine can occur. It should be pointed out, however, that in studies on

---

* This paper was presented at the meeting of the American Society of Biological Chemists at Atlantic City, March, 1948.

† Aided by a grant from the Pardee Foundation.

rabbits (11) and dogs (12) it was found that the sulfur of D-cystine is less readily oxidized than that of the L isomer. In order to test the availability of thiopyruvic acid for growth purposes, we synthesized the acid and administered it to growing rats orally or subcutaneously. No growth stimulation was observed, although under similar conditions L-cystine gave the usual growth response. Since thiopyruvic acid is readily oxidized *in vivo* to yield inorganic sulfate in the urine (13), these data seem to suggest that thiopyruvic acid, particularly the thiol group, is metabolized before the animal organism can utilize it for L-cysteine synthesis. Once the thiol group of thiopyruvic acid is stabilized by the benzyl group (as in S-benzylthiopyruvic acid), the synthesis of the L-cysteine derivative can and does take place.[1]

In discussions on N,N′-dimethylcystine (14) the argument was advanced that demonstration of the growth-promoting power of the methylated amino acid was tantamount to demonstrating that its keto analogue was also capable of supporting growth. The data presented here seem to suggest that the growth-promoting property of N,N′-dimethyl-L-cystine cannot definitely be ascribed to the formation of the keto analogue *in vivo*. Hydrolysis of the methyl groups *in vivo* to yield L-cystine appears the more likely possibility. Had the keto analogue been formed from N,N′-dimethyl-L-cystine, no growth would have resulted from its formation. It must be admitted, however, that it cannot be assumed *a priori* that the administration of thiopyruvic acid to the rat is equivalent to the situation when such a keto acid is formed in the tissues.

The method which we used for the synthesis of S-benzylthiopyruvic acid is based on that reported by Parrod (15). We found, however, that considerable simplification can be introduced in the procedure, particularly in the steps involving the use of sulfuryl chloride. It was also found that pyruvic acid can be directly chlorinated with sulfuryl chloride to yield chloropyruvic acid. A brief description of the methods of preparation of chloropyruvic and S-benzylthiopyruvic acids and their ethyl esters, and of sodium thiopyruvate, is presented. As far as we are aware, S-benzylthiopyruvic acid has not been previously prepared.

EXPERIMENTAL

Pyruvic Acid Ethyl Ester—33.2 gm. of pyruvic acid dissolved in 100 ml. of absolute ethanol which contained 0.3 ml. of concentrated sulfuric acid were refluxed for 3 hours. Excess ethanol was distilled off at atmospheric pressure on a water bath. The ester was collected, boiling at 44–45° at 10 mm. The yield was 50 to 60 per cent.

---

[1] In a private communication Dr. J. Parrod has just informed us that he isolated *p*-iodophenylmercapturic acid from the urine of rabbits which were fed S-*p*-iodophenylthiopyruvic acid. The synthesis of a cysteine derivative from a corresponding keto acid is thus not apparently confined to the organism of the rat.

Chloropyruvic Acid Ethyl Ester—60 gm. of pyruvic acid ethyl ester were dissolved in 220 ml. of sulfuryl chloride and the solution was heated on a water bath at 60–65° for 1.5 hours. The temperature was then gradually raised to 90–95° during the next 1.5 hours. Excess sulfuryl chloride was removed by distillation at atmospheric pressure. Chloropyruvic acid ethyl ester was collected at 79° at 9 mm. Parrod (15) reported that chloropyruvic acid ethyl ester boils at 90° at 15 mm. The yield was 60 to 70 per cent.

Chloropyruvic Acid—The compound was obtained by direct chlorination of pyruvic acid with sulfuryl chloride, as was described above. Chloropyruvic acid was collected, of boiling point 90–97° at 8 to 13 mm. The compound solidifies in a cool condenser and it is necessary to warm the condenser for efficient collection of the compound. Colorless shiny soft crystals were obtained. The compound is a strong vesicant. The yield was 50 to 65 per cent. For analysis it was dried at room temperature *in vacuo* over P_2O_5.

$C_3H_3O_3Cl$. Calculated. C 29.50, H 2.46, Cl 31.15
Found. " 29.06, " 2.55, " 31.60

S-Benzylthiopyruvic Acid Ethyl Ester—12.4 gm. of benzyl mercaptan were added to 100 ml. of water containing 5.6 gm. of KOH. With vigorous shaking of the solution, 15.1 gm. of chloropyruvic acid ethyl ester were added in several portions, the reaction mixture being kept alkaline (not below pH 9 to 10) by addition of extra pellets of KOH. The reaction vessel was kept immersed in an ice bath. After all of the chloropyruvic acid ethyl ester had been added, the flask was shaken for 15 to 20 minutes, allowed to stand at room temperature for 1 hour, then placed in the refrigerator for 3 hours. The mass which separated on standing was removed by decantation of the supernatant fluid and washed with cold water. The semisolid mass was then extracted with ether. The ether extract was washed with water, then dried with sodium sulfate. The ether was removed by distillation, and the residue was subjected once more to ether-water purification. The purified material was dried *in vacuo* at room temperature over P_2O_5. The compound remained a heavy oil after standing for several days at 2°.

$C_{12}H_{14}O_3S$. Calculated. C 60.50, H 5.88, S 13.44
Found. " 60.10, " 6.10, " 13.63

2,4-Dinitrophenylhydrazone of S-Benzylthiopyruvic Acid Ethyl Ester—0.2 gm. of 2,4-dinitrophenylhydrazine was dissolved in a little concentrated sulfuric acid and the solution was diluted with 9 volumes of absolute ethanol. To the solution 0.23 gm. of S-benzylthiopyruvic acid ethyl ester was added, and the mixture was gently warmed. On cooling, the crystals of the hydrazone separated which were collected and recrystallized three times from absolute ethanol. After being dried at 100° *in vacuo* over P_2O_5, the compound melted at 138–139° (uncorrected).

$C_{18}H_{18}N_4O_6S$.	Calculated.	C 51.67, H 4.31, N 13.39, S 7.66
	Found.	" 51.96, " 4.50, " 13.50, " 7.96

S-Benzylthiopyruvic Acid—To a solution of 13.2 gm. of benzyl mercaptan in 100 ml. of water containing about 8 gm. of KOH, 13 gm. of chloropyruvic acid were added in the manner described above. Upon completion of the reaction, the solution was extracted with ether, and the aqueous layer was acidified with HCl to pH 1 or 2. A semisolid mass separated almost immediately. It was removed by filtration, washed with cold water, then extracted with ether. The ether extract was dried with sodium sulfate, and the ether removed by distillation. The residue was further purified by extraction with ether, washing with water, drying as before, and distillation of the ether. The residue solidified at room temperature in a desiccator over sulfuric acid without formation of crystals.

$C_{10}H_{10}O_3S$.	Calculated.	C 57.14, H 4.76, S 15.24
	Found.	" 57.69, " 5.00, " 15.55

2,4-Dinitrophenylhydrazone of S-benzylthiopyruvic acid was prepared as described above. After drying *in vacuo* at 100° over P_2O_5, the compound melted at 148–149° (uncorrected).

$C_{16}H_{14}O_6N_4S$.	Calculated.	C 49.70, H 3.86, N 13.95, S 8.52
	Found.	" 49.23, " 3.60, " 14.30, " 8.20

Sodium Thiopyruvate—The compound was prepared essentially as was described by Parrod (15). On recrystallization from water by addition of ethanol the compound separated in the form of beautiful needles which contained 2 molecules of water of crystallization.

$NaC_3H_3O_3S \cdot 2H_2O$.	Calculated.	C 20.23, H 3.93, S 17.92
	Found.	" 20.40, " 4.25, " 17.86

Feeding Experiments

A complete 25 per cent casein diet which contained 1.0 per cent of S-benzylthiopyruvic acid was fed to four adult male rats of the Wistar strain. The rats had not previously been used for experiment. The urine was collected daily over a period of 5 days and preserved in a refrigerator. About 150 gm. of the food were consumed by the rats during this period. The pooled urine was then extracted in the manner described previously for the isolation of N-acetyl-S-benzyl-L-cysteine (1–3). The yield of the product obtained averaged 150 mg. per gm. of the keto acid ingested. In addition to N-acetyl-S-benzyl-L-cysteine, some unchanged S-benzylthiopyruvic acid was excreted by the rats. This was identified as the 2,4-dinitrophenylhydrazone, m.p. 148–149°. After drying *in vacuo* at 100° over P_2O_5, the isolated N-acetyl-S-benzyl-L-cysteine melted at 143–144°, and the melting point remained unchanged when the compound was mixed with an authentic sample of N-acetyl-S-benzyl-L-cysteine.

$C_{12}H_{15}O_3NS$.	Calculated.	C 56.92,	H 5.93,	N 5.54,	S 12.65,	Acetyl 16.98
	Found.	" 57.16,	" 6.20,	" 5.28,	" 12.43,	" 16.38

The isolated product had the specific rotation of $[\alpha]_D^{23} = -44°$ for a 1 per cent solution in ethanol.

S-Benzyl-L-cysteine was prepared from the isolated material by hydrolysis of the compound in dilute HCl in the usual manner and by precipitation of the cysteine derivative with ammonia. On recrystallization from water, S-benzyl-L-cysteine melted at 212–213° and had the specific rotation of $[\alpha]_D^{23} = +23°$ for a 1 per cent solution in N NaOH.

Growth Experiments

Three litters of albino male rats of Wistar strain were used. They were divided into three groups, six animals in each group. One group was fed an 8 per cent casein diet, the second the same diet supplemented with 1 per cent of sodium thiopyruvate, and the third group received 0.5 per cent of L-cystine as the supplement to the casein diet. The exact composition of the diet was described previously (16). After 6 weeks on these diets, the control group and the one ingesting thiopyruvic acid gained on the average of 1.2 gm. per day per rat, while the third group ingesting L-cystine gained on the average of 2.9 gm. per day per rat. Similar results were obtained when 75 mg. of sodium thiopyruvate in water were injected subcutaneously per rat per day in two equal portions 4 hours apart. It is thus evident that sodium thiopyruvate does not stimulate the growth of rats when administered either orally or subcutaneously under the conditions of our experiments.

SUMMARY

1. S-Benzylthiopyruvic acid was synthesized and fed to adult rats. From the urine of these animals N-acetyl-S-benzyl-L-cysteine was isolated and identified.

2. Sodium thiopyruvate does not stimulate the growth of rats when administered orally or subcutaneously in lieu of L-cystine.

3. It is concluded that the rat is able asymmetrically to synthesize S-benzyl-L-cysteine from the corresponding keto analogue. Since sodium

thiopyruvate did not stimulate the growth of rats in lieu of L-cystine, it appears that the keto acid is metabolized prior to its conversion to L-cysteine or L-cystine.

BIBLIOGRAPHY

1. Stekol, J. A., *J. Biol. Chem.*, **124**, 129 (1938).
2. Stekol, J. A., *J. Biol. Chem.*, **128**, 199 (1939).
3. Stekol, J. A., *J. Biol. Chem.*, **164**, 651 (1946).
4. du Vigneaud, V., Wood, J. L., and Irish, O. J., *J. Biol. Chem.*, **129**, 171 (1939). Binkley, F., Wood, J. L., and du Vigneaud, V., *J. Biol. Chem.*, **153**, 495 (1944).
5. Stekol, J. A., *Proc. Soc. Exp. Biol. and Med.*, **47**, 293 (1941).
6. Blanchard, M., Green, D. E., Nocito, V., and Ratner, S., *J. Biol. Chem.*, **155**, 421 (1944).
7. Shen, C.-W., and Lewis, H. B., *J. Biol. Chem.*, **165**, 115 (1946).
8. Stekol, J. A., *J. Biol. Chem.*, **140**, 827 (1941).
9. du Vigneaud, V., Dorfman, R., and Loring, H. S., *J. Biol. Chem.*, **98**, 577 (1932).
10. Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, **109**, 131 (1935).
11. du Vigneaud, V., Craft, H. A., and Loring, H. S., *J. Biol. Chem.*, **104**, 81 (1934).
12. Stekol, J. A., *J. Biol. Chem.*, **107**, 225, 641 (1934).
13. Parrod, J., *Comp. rend. Soc. biol.*, **141**, 132 (1947).
14. Kies, M. W., Dyer, H. M., Wood, J. L., and du Vigneaud, V., *J. Biol. Chem.*, **128**, 207 (1939).
15. Parrod, J., *Bull. Soc. chim.*, **14**, 109 (1947).
16. Stekol, J. A., and Weiss, K., *J. Biol. Chem.*, **175**, 405 (1948).

THE EFFECT OF RELATED IONS ON THE POTASSIUM REQUIREMENT OF LACTIC ACID BACTERIA*

By ROBERT A. MacLEOD and ESMOND E. SNELL

(*From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*)

(Received for publication, June 14, 1948)

The essential nature of K^+ for growth of lactic acid bacteria has previously been established (1). The amount required for maximum growth seemed unusually high. Further investigation showed that the quantitative requirement for potassium was markedly influenced by the amounts of sodium and ammonium ions in the medium. These findings prompted an extension of the investigation to other alkali metal ions. The results of these studies, which provide examples of "ion antagonism" among the alkali metals and which are most readily interpreted as due to competitive inhibition of the essential rôle of potassium by related ions, are presented below.

EXPERIMENTAL

Cultures and Inocula—*Lactobacillus arabinosus* 8014, *L. casei* 7469, *Leuconostoc mesenteroides* 8042 and 9135, and *Streptococcus faecalis* 8043 were carried as stab cultures in yeast-dextrose agar. Inoculum cultures were incubated 16 to 18 hours in a previously described medium (1). The cultures of *L. mesenteroides* 9135 were incubated at 30°, the remainder at 37°.

Basal Medium—To study the effect of the alkali metal and ammonium ions on the growth of the organisms, a medium as free as possible of these ions was prepared. A sulfuric acid hydrolysate of casein was used as the nitrogen source in the medium, the sulfate ion being removed with barium hydroxide (2). For rapid and heavy growth, acetate and phosphate are required. These are ordinarily employed as sodium or potassium salts, which was not desirable in this instance. To permit addition of these and other anions to the medium, preliminary experiments were run to determine the suitability of various amines as the base for neutralization of the medium. Of several tried, triethanolamine was found most satisfactory, since it was highly soluble and was non-toxic for the various test organisms at the concentrations required. The composition of the basal medium is given in Table I. The pH of the medium was adjusted to 6.8 with a 10 per cent

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from Eli Lilly and Company, Indianapolis, Indiana.

aqueous solution of triethanolamine. The amounts of Mn^{++} and Mg^{++} are considerably higher than those normally used in the presence of an acetate buffer (1). These amounts are required for rapid growth in the presence of triethanolamine, perhaps because triethanolamine, like citrate (1), forms complexes with these cations, thus lowering their effective concentrations.

TABLE I

Composition of Basal Medium

	Amount per 10 cc. final medium
	mg.
Acid-hydrolyzed casein*	100
Asparagine	1
DL-Tryptophan	0.5
Cystine	1
Adenine·HCl	0.1
Guanine·HCl	0.1
Uracil	0.1
Glucose	100
Acetic acid†	45
Phosphoric acid†	3
$MgSO_4 \cdot 7H_2O$	10
	γ
$MnSO_4 \cdot H_2O$	600
Pyridoxal·HCl	1
Thiamine	1
Calcium pantothenate	2
Riboflavin	2
Niacin	2
p-Aminobenzoic acid	1
Folic acid	0.05
Biotin	0.01

* See the text.

† The required amounts of acetic and phosphoric acids were neutralized with triethanolamine (Eastman Kodak, Practical) before addition to the medium. The same base was used to adjust the initial pH of the medium to 6.8.

This medium supported little or no growth of the organisms tested unless K^+ was added. Preliminary pretreatment to remove traces of K^+ (1) was thus omitted.

Test Procedure—The testing procedures were those customarily used in microbiological assay work and have been previously described (1). Unless otherwise specified, cultures were incubated for 24 hours, and metallic ions were added as the following salts: K^+, K_2SO_4; Na^+, Na_2SO_4; NH_4^+, $(NH_4)_2SO_4$; Rb^+, RbCl; Cs^+, CsCl; and Li^+, LiCl.

Sodium-Potassium Relationship—The effect of increasing levels of Na^+ on the growth response of *Lactobacillus casei* to K^+ is shown in Fig. 1. Sodium ion inhibits growth of this organism in the presence of low concentrations of K^+. The inhibitory action of Na^+ is completely reversed if sufficient K^+ is added. As the level of Na^+ is increased, the amount of K^+ required to prevent the resulting inhibition is also increased. Four other lactic acid bacteria showed similar behavior. The antibacterial indices for half maximum inhibition, *i.e.* the molar ratio of Na^+ to K^+ at which half maximum growth of each organism was obtained, are presented in Table II for several different concentrations of Na^+. Although the inhibitory effects of Na^+ are overcome in each case by additional quantities of K^+, the sensitivity of various organisms to the inhibitor (Na^+) varies considerably. The antibacterial indices are not entirely constant, but tend to increase with increasing concentration of Na^+. At the higher concentrations of Na^+ these ratios approach a constant value. The reasons for this variation are not clear; its magnitude, however, is similar to that frequently observed with organic metabolite-antimetabolite combinations (3). For *Leuconostoc mesenteroides* 8042 the inhibitory action of Na^+ at the concentrations tested is not due entirely to an uncomplicated antagonism involv-

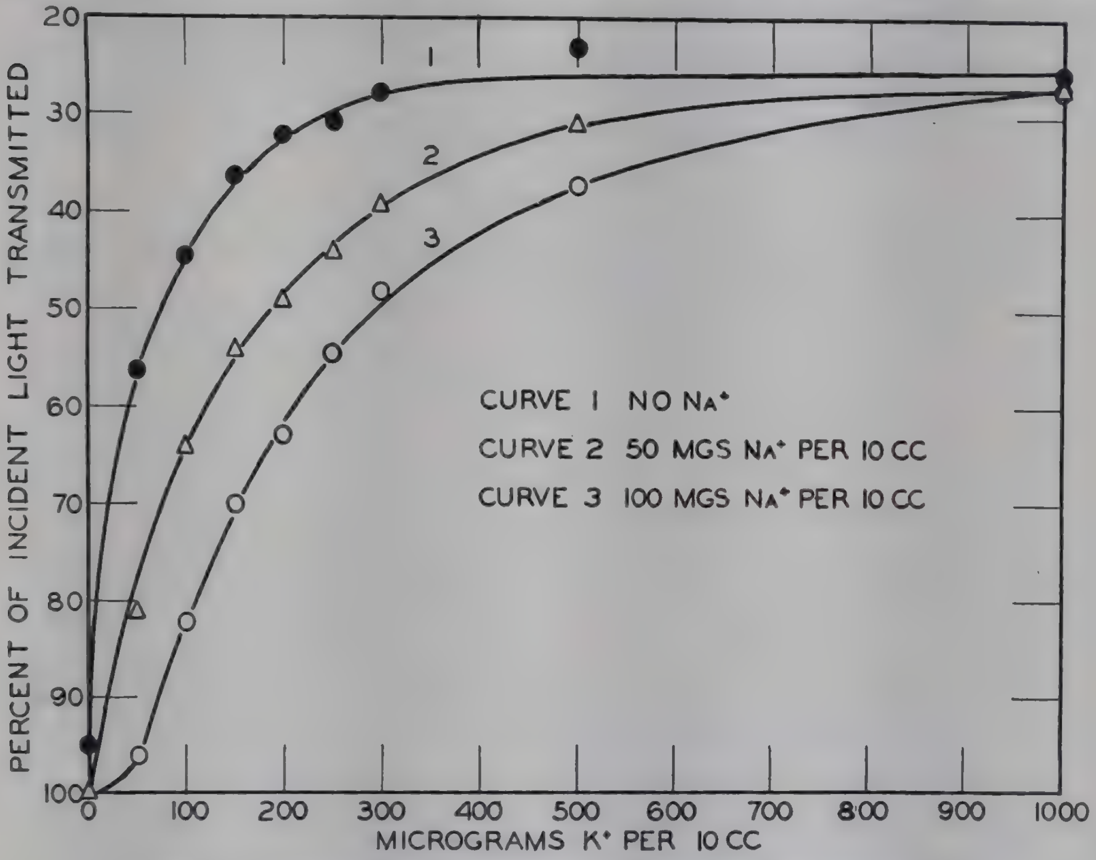


FIG. 1. Effect of Na^+ on response of *Lactobacillus casei* to K^+

ing K^+. The addition of large amounts of K^+ only partially reverses the inhibitory action of Na^+ for this organism. The nature of the inhibition not reversed by K^+ has not been investigated.

The same relation between Na^+ and K^+ concentrations apparent from Fig. 1, which presents data obtained following 24 hours of incubation, was found in separate trials after 120 hours incubation. The inhibitory effect of Na^+ is thus apparently not due solely to retardation of the rate of growth.

The highest concentration of Na^+ used, *i.e.* 100 mg., corresponds to approximately a 3 per cent solution of sodium sulfate. The question arose as to what extent the inhibition by Na^+ was due to the non-specific effects of high salt concentration (osmotic pressure effect, etc.) or to the effect of the anion associated with the salts. Fig. 2 shows the growth response of *Lactobacillus arabinosus* to very high concentrations of K^+, added as KCl and $\mathrm{K_2SO_4}$. Under our conditions neither salt is significantly inhibitory below a concentration of 200 mg. of K^+ per 10 cc. Above this concentration, the anion determines to a considerable extent the degree of inhibition. Plotted in this way, the toxicity of the sulfate ion appears much less than that of the chloride ion. This, however, is true because only half the molar concentration of $\mathrm{SO_4}^=$ as of Cl^- is required to furnish the same amount of K^+. On a molar basis $\mathrm{SO_4}^=$ and Cl^- are about equally toxic.

TABLE II

Molar Ratios of Na^+ to K^+ Which Permit Half Maximum Growth of Lactic Acid Bacteria at Various Levels of Added Na^+

Organism	Mg. Na^+ per 10 cc. medium			
	25	50	75	100
	Antibacterial index*			
Lactobacillus arabinosus	472	620	725	765
" *casei*	530	640	877	895
Streptococcus faecalis	340	530	688	805
Leuconostoc mesenteroides 8042	173	268	350	322
" " 9135		1890	2120	1990

* The antibacterial index is the molar ratio of inhibitor to metabolite (in this case Na^+ to K^+) at which growth is reduced to one-half of the maximum.

These experiments indicate that, since Na^+ was added as the sulfate and at concentrations not exceeding 100 mg. per 10 cc., the observed inhibition of *Lactobacillus arabinosus* was not to any appreciable extent due either to high salt concentration or to the presence of the anion. At concentrations considerably higher than those used here, sodium salts will, of course, become toxic due to osmotic and perhaps other effects, and such toxicity will not be alleviated by additions of K^+. At the concentrations used in this investigation, however, the principal toxic action of Na^+ appears to be due to its interference with K^+ utilization, and is prevented competitively by additions of K^+.

These results prompted investigation of the possible relationship of other alkali metal ions and of NH_4^+ to the requirement for K^+. These results are summarized below.

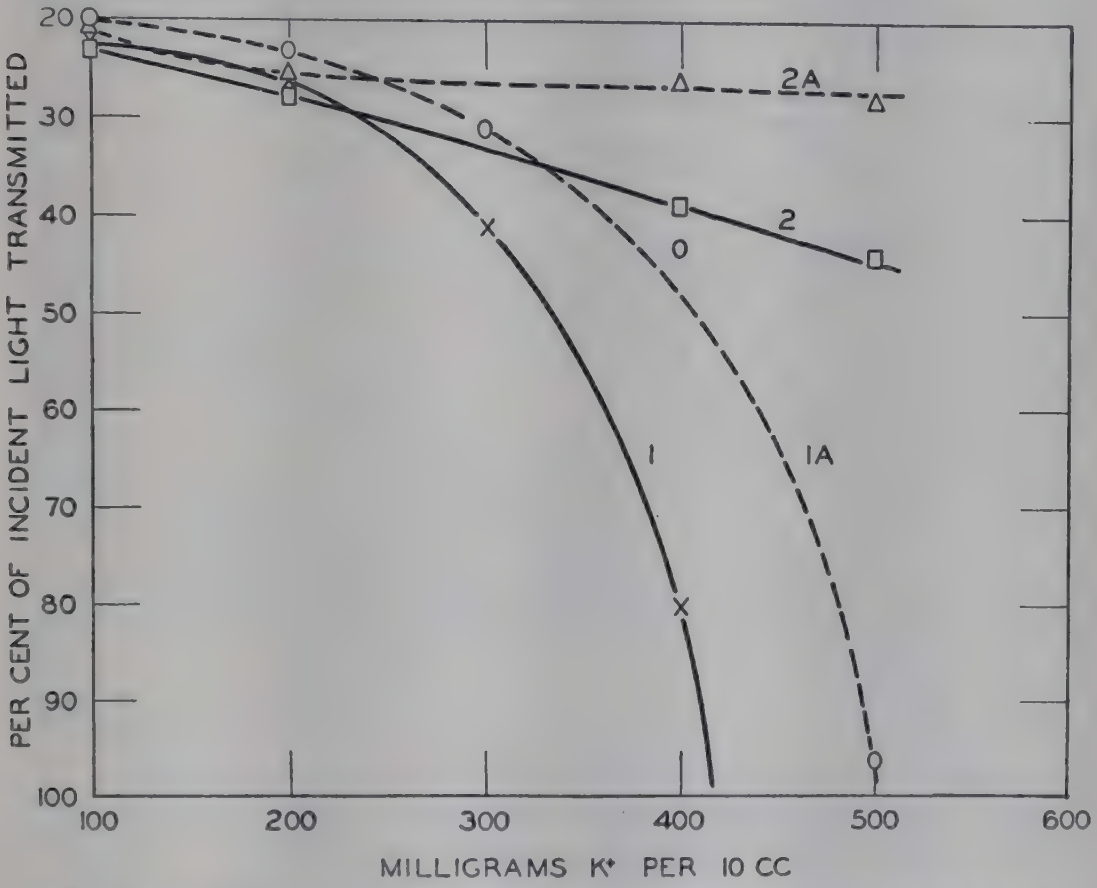


FIG. 2. Inhibitory effects of high concentrations of potassium salts on growth o *Lactobacillus arabinosus*. Curve 1, KCl, 24 hours incubation; Curve 1A, KCl, 48 hours incubation; Curve 2, K_2SO_4, 24 hours incubation; Curve 2A, K_2SO_4, 48 hours incubation.

Ammonium-Potassium Relationship—The effect of NH_4^+ on the response of five lactic acid bacteria to K^+ was determined. The results obtained with *Lactobacillus casei* are presented in Fig. 3. The ammonium ion, like Na^+, is inhibitory to growth, and the inhibition is alleviated by increasing amounts of K^+. The concentration of K^+ required to reverse the inhibition increases as the amount of NH_4^+ increases. A similar relationship was observed with the other four organisms tested. The ratios of the concentration of NH_4^+ to K^+, at which half maximum inhibition of growth of the five lactic acid bacteria occurs, are shown in Table III. As with Na^+, the lowest concentration of NH_4^+ gives a lower value for this ratio than do higher levels. The remaining ratios are much more nearly constant for each organism than are those for Na^+, and indicate the competitive nature of the inhibition. The values obtained with *Leuconostoc mesenteroides* 8042 show the greatest variation, though for this organism, too, the toxicity of NH_4^+ is almost completely overcome by K^+. Comparison of the antibacterial indices for NH_4^+ (Table III) and Na^+ (Table II) shows that NH_4^+ is considerably more effective than Na^+ in counteracting the growth-promoting effects of K^+.

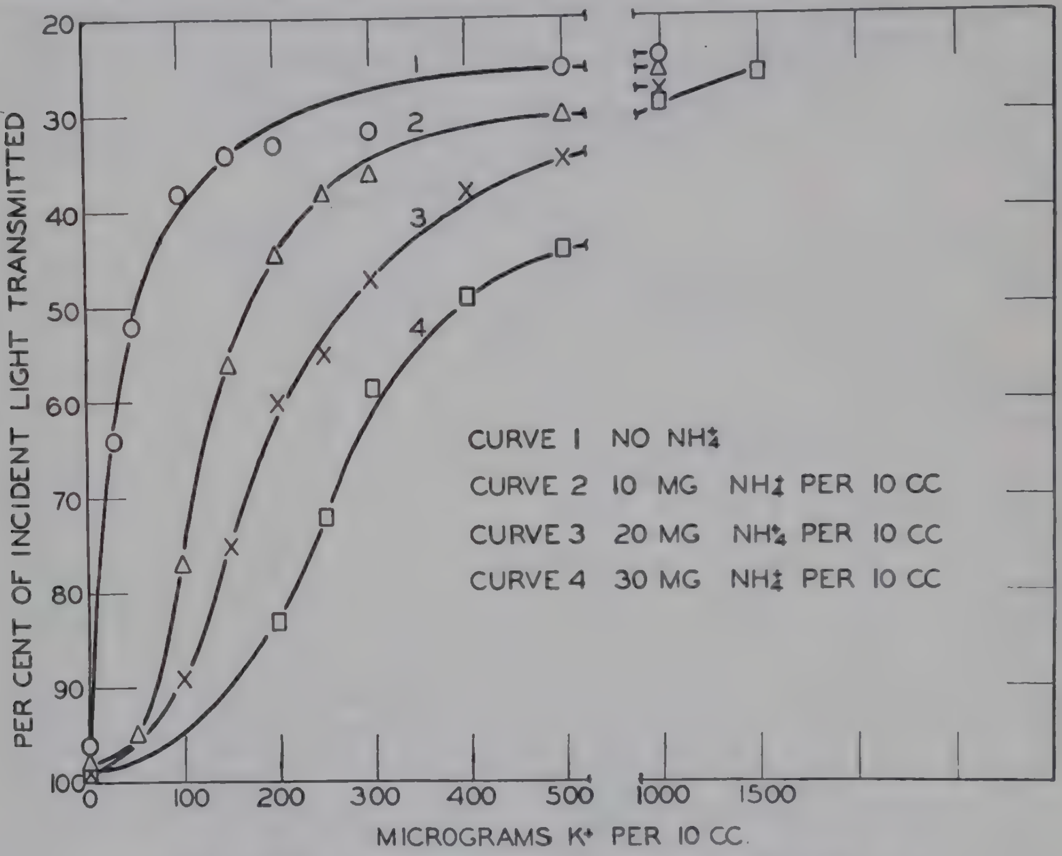


FIG. 3. Effect of NH_4^+ on response of *Lactobacillus casei* to K^+

To determine whether the relationship observed above for lactic acid bacteria holds more generally, the effect of Na^+ and NH_4^+ on the response of the yeast, *Saccharomyces carlsbergensis* 4228, to K^+ was determined. The medium (Table I) was supplemented with Ca^{++} (1 mg. per 10 cc.), inositol (250 γ per 10 cc.), Fe^{++} (10 γ per 10 cc.), and the pH adjusted to 5.5 to support more favorably the growth of this organism. Cultures were shaken mechanically at 30° for 24 hours. From Table IV it is evident that both ions interfere with the utilization of K^+ by the yeast in a manner analogous to their effect in lactic acid bacteria. In contrast to the response of the bacteria, however, the inhibitory effect of Na^+ is greater than that of NH_4^+ for yeast. The yeast is considerably more sensitive to Na^+ than are the lactic acid bacteria tested.

These findings permit a partial explanation for the high potassium requirement previously noted (1) for lactic acid bacteria. This requirement was determined in a medium which contained considerable amounts of sodium and ammonium ions; the "potassium requirement" observed represented not only that required for metabolic processes, but also that required to overcome the inhibitory action of Na^+ and NH_4^+. The requirement for potassium, as determined in the present medium, is about one-fifth of that previously observed. It is not at all certain, however, that this represents solely the metabolic requirement for this ion. Other ions in the present complex basal medium may enhance the requirement for K^+, just as did Na^+ and NH_4^+ in the previously used medium.

TABLE III

Molar Ratios of NH_4^+ to K^+ Permitting Half Maximum Growth at Various Levels of Added NH_4^+

Organism	Mg. NH_4^+ per 10 cc. medium			
	10	20	30	40
	Antibacterial index*			
Lactobacillus arabinosus	115	135	149	140
" *casei*	176	238	238	230
Streptococcus faecalis	88	105	112	115
Leuconostoc mesenteroides 8042	79	144	178	204
" " 9135	230	268	280	208

* As in Table II.

TABLE IV

Effect of Na^+ and NH_4^+ on Response of Saccharomyces carlsbergensis 4228 to K^+

K^+ per 10 cc.	Mg. Na^+ per 10 cc.				Mg. NH_4^+ per 10 cc.		
	0	25	50	75	25	50	75
	Per cent of incident light transmitted*						
γ							
0	93	100	100	100	100	99	100
100	37	92	100	99	98	98	100
1,000	15	25	79	95	26	42	79
10,000	15	16	27	45	17	22	30

* Evelyn colorimeter, 660 mμ filter, uninoculated medium = 100.

It was of interest to determine whether the triethanolamine ion, which replaces Na^+ as the principal neutralizing cation in this medium, interfered to any extent with the utilization of K^+. For this purpose, extra triethanolamine was added to the medium as the hydrochloride, with results shown in Table V. Since 175 to 200 mg. of triethanolamine per 10 cc. are used in the preparation of the medium, the amounts added do not represent the total concentration of this base in the medium. Only after 200 mg. of triethanolamine have been added in addition to that already present in the basal medium is there any appreciable toxicity for *Lactobacillus arabinosus*. Since significant increases in triethanolamine concentration have so little effect on the response to K^+, it is reasonable to assume that the amounts originally present in the medium interfere very little in the utilization of this ion. At high concentrations of the amine, however, some degree of antagonism does exist between K^+ and the triethanolamine ion, since inhibition at low concentrations of K^+ is almost completely reversed at high. At still higher concentrations of triethanolamine, however, the inhibition is not prevented by K^+.

TABLE V

Effect of Triethanolamine on Response of Lactobacillus arabinosus to K^+

K^+ per 10 cc.	Mg. triethanolamine per 10 cc.*				
	0	100	200	300	400
	Per cent of incident light transmitted†				
γ					
0	90	91	98	100	100
50	66	65	69	73	97
100	52	53	58	59	90
1000	21	22	23	27	44

* The amounts indicated are in addition to the triethanolamine used in the preparation of the medium (about 175 to 200 mg. per 10 cc.; see the text).

† As in Table IV.

Rubidium-Potassium Relationship—The response of the five lactic acid bacteria to additions of Rb^+ to the potassium-free basal medium was determined. On a molar basis, Rb^+ is slightly more active in promoting growth of *Streptococcus faecalis* than K^+ at low concentrations and slightly less so at high (Fig. 4). The results of similar experiments with *Lactobacillus casei* and *Leuconostoc mesenteroides* 8042 are presented in Fig. 5. For *L. casei*, Rb^+ and K^+ are equally active at low concentrations; at higher concentrations, Rb^+ again is less active. *L. mesenteroides* 8042, by contrast, is unable to utilize Rb^+ in place of K^+. In similar experiments, *L. arabinosus* and *L. mesenteroides* 9135 proved intermediate in behavior. For

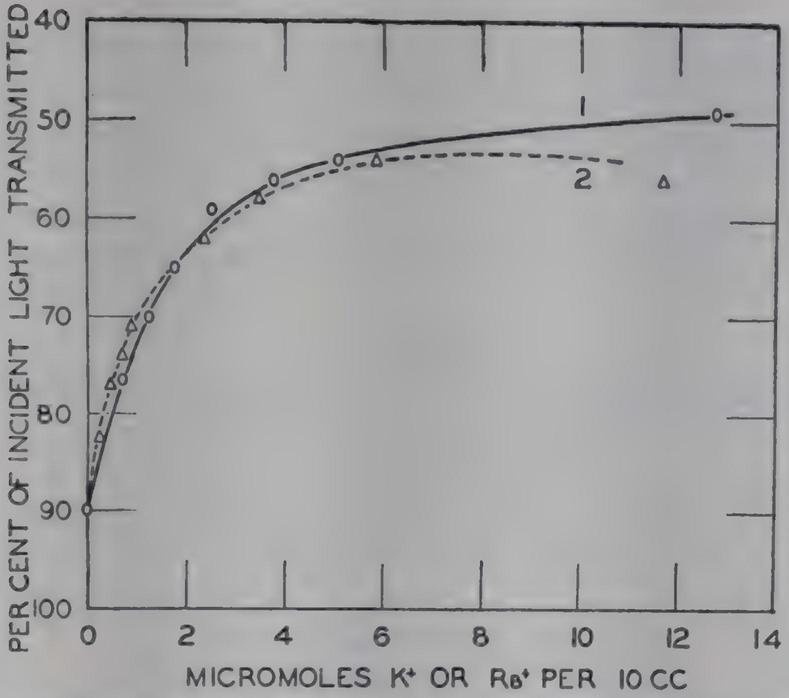


FIG. 4. Comparative effects of Rb^+ and K^+ on growth of *Streptococcus faecalis*. Curve 1, K^+; Curve 2, Rb^+.

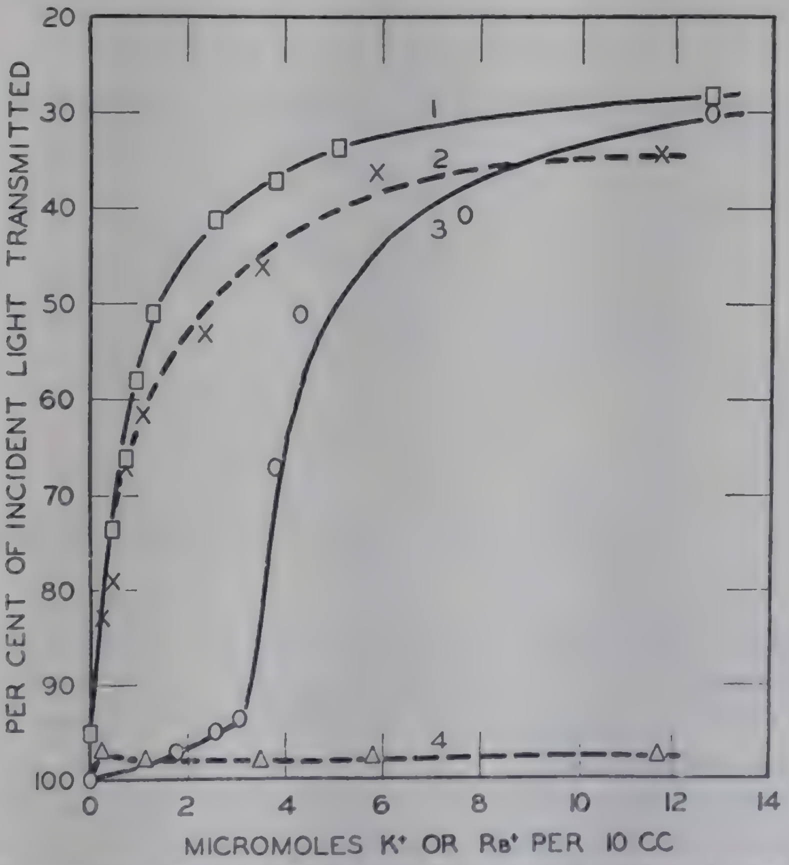


FIG. 5. Comparative effects of Rb^+ and K^+ on growth of *Lactobacillus casei* and *Leuconostoc mesenteroides* 8042. Curves 1 and 2, response of *L.* casei to K^+ and Rb^+ respectively; Curves 3 and 4, response of *L. mesenteroides* to K^+ and Rb^+ respectively

these organisms, Rb^+ partially replaced K^+, but the response was erratic. More than half maximum growth for either organism was never achieved with Rb^+ and high concentrations were inhibitory. Thus, depending upon the organism, Rb^+ is able to replace K^+ completely, partially, or not at all.

High concentrations of Rb^+ are inhibitory for *Leuconostoc mesenteroides* 8042 and the inhibition is reversed by the addition of sufficient K^+ (Table VI). A similar relationship was found with *L. arabinosus*, despite the fact that at low concentrations Rb^+ partially replaces K^+. In organisms for which it is not the nutritional equivalent of K^+, Rb^+ thus acts in a manner analogous to Na^+ and NH_4^+.

TABLE VI

Effect of K^+ on Inhibitory Action of Rb^+ for Leuconostoc mesenteroides 8042

K^+ per 10 cc.	Mg. Rb^+ per 10 cc.			
	0	10	20	30
	Per cent of incident light transmitted*			
γ				
0	97	90	93	93
500	29	60	84	89
1,000	28	45	64	84
20,000	30	30	30	31

* As in Table IV.

TABLE VII

Effect of Rb^+ on Inhibitory Action of NH_4^+ for Lactobacillus casei

Rb^+ per 10 cc.	Mg. NH_4^+ per 10 cc.		
	0	20	40
	Per cent of incident light transmitted*		
γ			
0	67	89	90
100	46	78	89
500	29	51	66
1,000	25	47	56
10,000	33	24	25

* As in Table IV. The antibacterial index ($[NH_4^+]/[Rb^+]$) for half maximum growth calculated from these data is about 350.

For organisms which utilize Rb^+ in place of K^+, factors which interfere with the response to K^+ would be expected to interfere similarly with the response to Rb^+. The data of Table VII show that the inhibitory action of NH_4^+ for *Lactobacillus casei* is alleviated by Rb^+, just as it is by K^+.

The ratio of NH_4^+ to K^+ permitting half maximum growth is about 230 (Table III); the corresponding ratio of NH_4^+ to Rb^+ is about 350. This indicates that NH_4^+ is more toxic in the presence of K^+ than in the presence of the same molar concentration of Rb^+; *i.e.*, Rb^+ is somewhat more effective in overcoming the inhibitory action of NH_4^+ than is K^+. This must indicate a greater affinity of this metallic ion for the enzyme with which it functions than K^+ possesses.

TABLE VIII

Effect of K^+ on Inhibitory Action of Cs^+ for Lactobacillus arabinosus

K^+ per 10 cc.	Mg. Cs^+ per 10 cc.			
	0	10	30	50
	Per cent of incident light transmitted*			
γ				
0	80	95		
500	25	24	38	82
1,000	19	23	26	43
10,000	20	20	21	22

* As in Table IV.

TABLE IX

Effect of K^+, Na^+, and NH_4^+ on Li^+ Toxicity for Lactobacillus arabinosus

	Mg. per 10 cc.				
K^+	0	100	10	10	10
Na^+	0	0	10	0	10
NH_4^+	0	0	0	10	10
Mg. Li^+ per 10 cc.	Per cent of incident light transmitted*				
0	94	32	32	32	26
10	98	96	65	33	32
15	98	98	95	57	35
20	98	97	96	97	72

* As in Table IV.

Cesium-Potassium Relationship—Cesium ion did not replace K^+ for the five organisms tested. For *Lactobacillus arabinosus*, Cs^+ was inhibitory at high concentrations. This inhibition was completely reversed by the addition of sufficient K^+ (Table VIII).

Lithium-Potassium Relationship—For *Lactobacillus arabinosus*, Li^+ was considerably more toxic than either Na^+ or NH_4^+. This toxicity was not alleviated by K^+. The addition of Na^+ relieved the inhibition slightly; NH_4^+ was somewhat more effective, and the two together were more effective than either alone (Table IX). These results suggest that both Na^+

and NH_4^+ ions play some essential metabolic rôle in *Lactobacillus arabinosus*, and that part of the toxic effects of Li^+ results from interference with these processes. No direct evidence has been obtained to show that Na^+ is essential in the metabolism of these organisms. It is obvious, however, that NH_4^+ must play a rôle in intermediary metabolism in these bacteria, and some direct evidence for this, obtained by growth effects (4, 5) exists.

DISCUSSION

The term "ion antagonism" has been used to describe the opposite effect of ions or the effect of an "inactive" ion in removing the effect of an "active" one (6). The phenomenon was first observed by Ringer (7), who found that a solution of sodium chloride does not maintain the beat of a heart perfused with it unless additions of Ca^{++} and K^+ chlorides are made. The concept of ion antagonism was applied to animals by Loeb (8), to plants by Osterhout (9), and to bacteria by Flexner (10), Eisler (11), and Lipmann (12). Since these early investigations, many further observations regarding the phenomenon have been reported (6).

Although it has been recognized recently that animals may be deficient in one mineral relative to an excess of some other (13), none of the early data on ion antagonism was able to relate the phenomenon to the inorganic requirements of the organism under investigation. Loeb discounted the possibility that physiologically balanced solutions had nutritional significance for a number of organisms and favored the hypothesis that such solutions were required to maintain proper cell permeability (14).

The antagonistic effects of ions observed in this investigation are most easily explained as examples of the competitive interference of one ion with the essential rôle of another. This conclusion is based on the following observations: (1) the inhibition is reversed only by an inorganic ion which is an essential metabolite for the microorganism; (2) the inhibition is apparent only in the presence of limiting concentrations of the metabolite and is reversed, in most cases completely, if the metabolite concentration is raised sufficiently; (3) the ratio of antagonist to metabolite which permits a given amount of growth is relatively constant over a considerable range of concentrations.

It is usually assumed that inhibitions of this type result from the reversible combination of a metabolite or its analogue with some active surface or enzyme in or on the cell whose proper functioning is essential for growth. Whether enzyme is present as the active enzyme-metabolite complex or as the inactive enzyme-antimetabolite complex thus depends upon the relative concentration of metabolite to antimetabolite, and not upon their absolute concentrations. The factors affecting the affinity of a metallic ion for an enzyme are obscure. The fact that Rb^+ can replace K^+ for one organism and antagonize its action for another indicates subtle though critical differences in the structure of enzymes having the same or a similar function. In both cases, however, the combination of Rb^+ with the enzyme is indicated. That Rb^+ can substitute for K^+ in isolated enzymatic processes dependent upon the presence of the latter ion was shown by Lwoff and Ionesco (15), who showed that either Rb^+ or Cs^+ could function in place of K^+ in permitting production of pyruvate from malate by cell preparations of *Moraxelli lwoffi.*

Previous figures reported as the K^+ requirement for maximum growth of lactic acid bacteria ranged from 1 to 10 mg. per 10 cc. (1). On the basal medium used here, in the absence of Na^+ or NH_4^+, maximum growth can be obtained with amounts of K^+ ranging from 0.2 to 1 mg. per 10 cc. It is apparent that the concentration of an essential ion required for growth, or the concentration of an ion which will inhibit growth, is not a constant but may vary widely, depending upon the composition of the medium in which the determinations are made.

The practical significance of these observations should not be overlooked. Considerable and variable quantities of Na^+ and NH_4^+ are present in media used for microbiological assay of vitamins and amino acids. Furthermore, if sodium chloride is added with a hydrolyzed sample, the amount present will vary from one assay tube to another. In the absence of sufficient amounts of K^+, inhibitory concentrations of Na^+ may well be reached. The use of potassium hydroxide in place of sodium hydroxide for neutralization of various components of the medium might be expected to eliminate these sources of error. Such a medium was found to support excellent growth of the five organisms used in this investigation. Observations concerning the relative toxicities of sulfate and chloride ions suggest that sulfuric acid might prove superior to hydrochloric acid in the preparation of samples for assay.

SUMMARY

Preparation of a medium which is deficient in K^+ and relatively free of NH_4^+ and Na^+ is described. Triethanolamine was used for neutralization of the acidic components of this medium. The medium supports excellent growth of the five lactic acid bacteria tested when supplemented with adequate amounts of K^+.

In this medium, it was shown that the magnitude of the K^+ requirement of all organisms investigated was greatly increased by the addition of Na^+ and NH_4^+. Whether or not these ions inhibited growth depended upon the ratio of their concentrations to that of K^+, and not upon the absolute amounts present. For a given amount of growth these ratios were relatively constant over a fairly wide range of concentrations; *i.e.*, a competitive

relationship between these ions and the essential metabolite, K^+, appeared to be present.

Rubidium ion replaced K^+ completely for growth of *Lactobacillus casei* and *Streptococcus faecalis*, partially for *L. mesenteroides* 9135 and *L. arabinosus*, and not at all for *Leuconostoc mesenteroides* 8042. For the latter two organisms Rb^+ was inhibitory at higher concentrations and the inhibition was competitively alleviated by K^+. For *L. casei*, which utilizes Rb^+ in place of K^+ for growth, Rb^+ was slightly more effective than K^+ in reversing inhibition of growth by NH_4^+.

Cesium ion did not replace K^+ as an essential metabolite for any of these organisms. For *Lactobacillus arabinosus*, the only organism investigated, Cs^+ was inhibitory at high concentrations, and this inhibition was alleviated by K^+.

In contrast to the other alkali metal ions, toxicity of Li^+ for *Lactobacillus arabinosus* is not related to the K^+ requirement of the organism. Inhibition of growth by Li^+ is prevented over a narrow range of concentrations by the addition of small amounts of NH_4^+ and Na^+.

Certain of these results have been discussed briefly.

BIBLIOGRAPHY

1. MacLeod, R. A., and Snell, E. E., *J. Biol. Chem.*, **170,** 351 (1947).
2. *Univ. Texas Pub., No. 4137*, 82 (1941).
3. Shive, W., and Macow, J., *J. Biol. Chem.*, **162,** 451 (1946).
4. Hac, L. R., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **159,** 273 (1945).
5. Waelsch, H., Owades, P., Miller, H. K., and Borek, E., *J. Biol. Chem.*, **166,** 273 (1946).
6. Falk, I. S., *Abstr. Bact.*, **7,** 33, 87, 133 (1923).
7. Ringer, S., *J. Physiol.*, **3,** 380 (1880–82); **4,** 29, 222 (1882–83).
8. Loeb, J., *J. Biol. Chem.*, **1,** 427 (1905–06).
9. Osterhout, W. J. V., *Bot. Gaz.*, **42,** 127 (1906).
10. Flexner, S., *J. Exp. Med.*, **9,** 105 (1907).
11. Eisler, M., *Centr. Bakt. 1. Abt., Orig.*, **51,** 54 (1909).
12. Lipmann, C. B., *Bot. Gaz.*, **48,** 105 (1909).
13. Smith, S. E., and Larson, E. J., *J. Biol. Chem.*, **163,** 29 (1946).
14. Loeb, J., The mechanistic conception of life, Chicago (1912).
15. Lwoff, A., and Ionesco, H., *Compt. rend. Acad.*, **225,** 77 (1947).

A SIMPLE APPARATUS FOR THE SERIAL MEASUREMENT OF THE RESPIRATORY EXCHANGE IN THE RAT

BY DAVID LESTER AND LEON A. GREENBERG

WITH THE TECHNICAL ASSISTANCE OF RICHARD P. CARROLL

(From the Laboratory of Applied Physiology, Yale University, New Haven)

(Received for publication, June 18, 1948)

For the frequent measurement of the respiratory exchange of small animals over successive short periods of time, all of the methods described present difficulties. In the open circuit method determination of oxygen consumption over a short interval of time is not practical. In the closed circuit methods considerable time is required for temperature equilibrium to be attained before a measurement can be made. Both carbon dioxide and oxygen analyses are necessary in the closed non-ventilated chamber method.[1]

In the method described here neither the maintenance of a constant temperature nor analysis for oxygen is required, thus facilitating the measurement of the respiratory exchange over successive short time intervals. This method is based on a principle which, to our knowledge, has never been described before.

The apparatus is a closed, non-ventilated chamber in which atmospheric pressure is maintained by means of a small spirometer. For the calculation of the respiratory exchange, the data needed are the initial and final temperatures, barometric pressure, and the percentage of carbon dioxide. In addition, the free volume of the chamber must be known and the system must be maintained at 100 per cent humidity. The oxygen consumption is calculated as the carbon dioxide production plus the change in volume of the system.

Fig. 1 is a schematic diagram of the system. *A* is a glass desiccator having three outlets in its cover; the free volume of this chamber is 3300 cc. *B* is a small fan, *C* and *F* in Fig. 1 are outlet and inlet tubes for flushing the chamber, *D* is the power supply cable for the fan motor, *G* is an iron-constantan thermocouple for measurement of the rectal temperature of the rat, and *H* is a thermometer (0–50°) readable to 0.02°. S_1 is the 2-way cock in the capillary tube to the carbon dioxide gas analyzer whose accuracy is 0.02 per cent. Tube *E* leads to the water manometer (*J*) and the small test-tube spirometer (*K*), which has a volume of about 70 cc. and is counterbalanced by the weight (*L*). The spirometer is connected through a 2-way cock (S_2) to a 50 cc. glass syringe (M) which is read to 0.5 cc. Preliminary to a series of determinations approximately 5 cc. of 10 per cent sulfuric acid are placed in the desiccator to obviate the absorption of any appreciable amounts of carbon dioxide.

---

[1] Benedict, F. G., in Abderhalden, E., Handbuch der biologischen Arbeitsmethoden, Berlin and Vienna, Abt. IV, Teil 10, 427 (1924).

The respiratory exchange is determined by the following procedure. The rat is placed on a perforated porcelain support in the desiccator and the cover is affixed.[2] After E is clamped off and S_1 is closed, air saturated with water at 36° is blown through the chamber at about 25 liters per minute through F and exhausted at C. This rate of flushing is sufficient to prevent any detectable accumulation of carbon dioxide. During the process of flushing the counterpoise is disconnected and the spirometer is brought to its lowest level and adjusted to atmospheric pressure with the syringe. When the manometer indicates atmospheric pressure, S_2 is turned to room air and 50 cc. of air are admitted into the syringe. The room temperature and the volume of air are recorded. S_2 is then connected with the spirometer and the air in the syringe is admitted to it. At the time chosen to start the experimental period, the air flow is stopped, F and C are clamped, E is unclamped, and the temperature of the chamber and the barometric pressure are recorded.

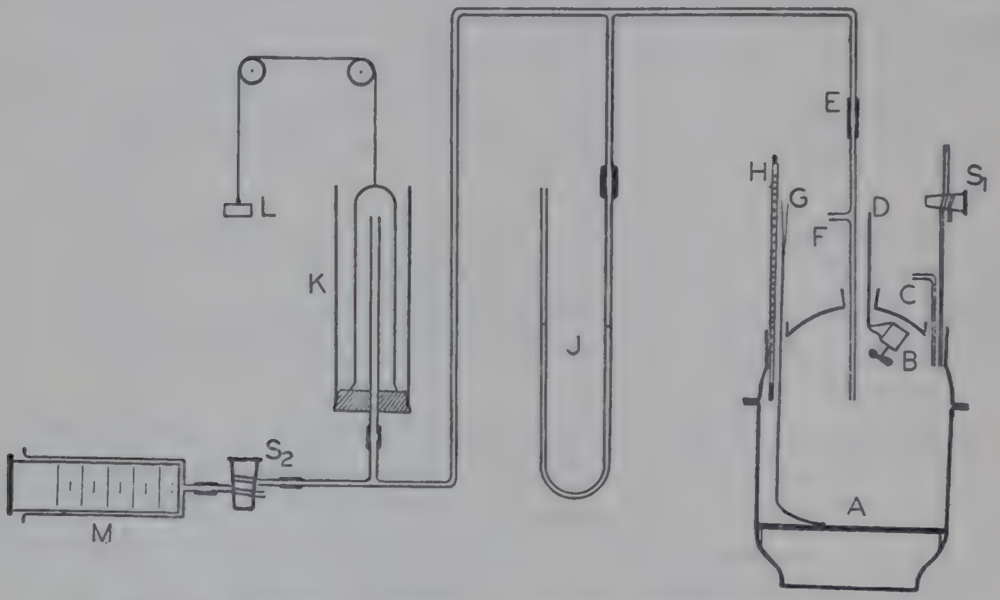


FIG. 1. Schematic diagram of the respiratory apparatus

About 1½ minutes before the period is over the fan is started to attain temperature equilibrium. Some 7 seconds before the end of the period the fan is stopped and the spirometer is brought to atmospheric pressure by means of the syringe or the counterpoise. At the end of the period, E is clamped and the temperature of the chamber and the barometric pressure are read. The fan is started again, S_1 is opened, and a sample of air from the chamber is drawn for carbon dioxide analysis. The time elapsed between the end of the experimental period and the final drawing of the air sample is noted. The fan is stopped, S_1 is closed, C and F are unclamped, and the flushing is begun again. The volume of air left in the spirometer is measured by the syringe and this volume and the room temperature recorded.

---

[2] Dow-Corning high vacuum type Silicone stop-cock grease gives an air-tight seal more conveniently than other greases tried.

50 cc. of air are again admitted into the spirometer. The analysis for carbon dioxide is performed and recorded and the apparatus is now ready for another determination. During the second experimental period the results of the first period may be calculated.

With a room temperature of approximately 24° the temperature of the chamber is maintained close to the critical temperature of the rat (28°). During an experimental period the temperature changes no more than 0.4°. The data obtained are recorded as follows: b.p. = barometric pressure; $V_{ch.}$ = free volume of chamber; $T^1{}_{ch.}$, $T^2{}_{ch.}$ = initial and final temperature in chamber; $T^1{}_{rm.}$, $T^2{}_{rm.}$ = initial and final temperature of room; $V^1{}_{s.}$, $V^2{}_{s.}$ = initial and final volume of syringe; and $W^1{}_{ch.}$, $W^2{}_{ch.}$, $W^1{}_{s.}$, $W^2{}_{s.}$ = vapor pressure of water in chamber and spirometer at observed temperatures. The dry gas volumes at standard temperature and pressure initially (V^1) and finally (V^2) are

$$V^1 = \left[\frac{\text{b.p.} - W^1{}_{ch.}}{T^1{}_{ch.} + 273.18} \times V_{ch.} \times \frac{273.18}{760}\right] + \left[\frac{\text{b.p.} - W^1{}_{s.}}{T^1{}_{rm.} + 273.18} \times V^1{}_{s.} \times \frac{273.18}{760}\right]$$

and V^2 is similarly calculated. The percentage of carbon dioxide found (less 0.03 per cent for that present in atmospheric air) is adjusted for the time period used: If the period is 15 minutes and the air sample from the chamber is obtained at 15 minutes and 30 seconds, the percentage of carbon dioxide produced per 15 minutes is 15/15.5 of that actually found by analysis. The amount of carbon dioxide produced is then

$$\text{Cc. } CO_2 = \%CO_2 \times V^2$$

and the amount of oxygen consumed is

$$\text{Cc. } O_2 = \text{cc. } CO_2 + (V^1 - V^2)$$

If the R.Q. is unity, there will be no change in volume of the system, since equal amounts of carbon dioxide are produced and oxygen consumed and V^1 will be equal to V^2. If the R.Q. is less than unity, V^2 will be less than V^1 by an amount equal to the difference between the oxygen consumed and the carbon dioxide produced.

The time-separating experimental periods need be no longer than 5 minutes, so that three 15 minute periods of oxygen consumption and carbon dioxide production may be measured by one operator in 1 hour and the requisite calculations performed.

As in all measurements of the respiratory exchange of small animals, the system must be air-tight. This feature is tested occasionally by increasing and decreasing the pressure within the system by means of the syringe (the spirometer being clamped off) and observing the manometer. In addition a blank run is conducted; the calculated V^1 should not differ from V^2 by more than about 0.2 per cent.

The accuracy of the entire procedure was tested by the combustion of two pure, organic compounds. For this purpose the thermocouple was replaced by a power cable connected within the chamber to a short length of platinum wire. The platinum wire is placed in contact with a glass wool wick which protrudes about 2 mm. from a short length of heavy capillary tubing (7 mm. outer diameter × 20 mm.). The capillary is held in glass to glass contact by means of a short length of rubber tubing with a small shell vial (9 mm. outer diameter × 30 mm.) which holds the liquid to be combusted and into which the wick extends. The combustion lamp is held in place within the chamber by means of a rubber stopper. The calculated volume of these appurtenances is subtracted from the free volume of the chamber.

TABLE I

Ethyl Alcohol

R.Q. = 0.667.

CO_2 produced	O_2 consumed	R.Q.	Error in R.Q.
cc.	*cc.*		*per cent*
79.5	119.8	0.664	−0.40
85.6	131.8	0.649	−2.65
83.7	126.2	0.663	−0.55
175.6	268.5	0.654	−1.90
165.0	251.3	0.657	−1.45
192.5	291.5	0.660	−1.00

The procedure for measurement is essentially the same as that described for the rat. The lamp is lighted by momentarily heating the platinum to just less than white heat after the chamber has been sealed at S_1, C, and F, and opened at E. The spirometer is left at its lowest level to allow for the initial, rapid expansion of volume that takes place from the rise in temperature as a result of the combustion. The combustion can be terminated at any time by starting the fan. The amount of material that can be burned is determined by the initial concentration of oxygen. To burn greater amounts of material, air enriched in oxygen (about 28 per cent oxygen) and saturated with water is flushed through the chamber. After the lamp is extinguished, the temperature within the chamber is allowed to fall to within 0.5° of the initial temperature before the final readings are made. This insures complete saturation within the chamber.

With this technique, 95 per cent ethyl alcohol was burned; the results are shown in Table I. The loss of ethyl alcohol, by volatilization, is great, due to the flushing of the chamber and heating of the platinum. This precludes calculation of the theoretical oxygen consumption, but not of the R.Q. No error is introduced by the evaporation of ethyl alcohol, since practically all of it is dissolved in the water of the chamber and does not appear as gas.

TABLE II

Diethylene Glycol

R.Q. = 0.800.

Amount combusted	CO_2 produced	O_2 consumed	R.Q.	Error in CO_2	Error in O_2	Error in R.Q.
mg.	*cc.*	*cc.*		*per cent*	*per cent*	*per cent*
116.6	97.0	124.2	0.781	−1.52	+0.89	−2.37
118.8	98.8	128.4	0.769	−1.59	+2.31	−3.87
303.0	250.2	320.0	0.782	−2.27	0.00	−2.25
257.7	212.4	271.1	0.783	−2.43	−0.37	−2.11
137.0	119.1	153.1	0.778	+2.94	+5.81	−2.75
284.9	243.3	310.1	0.785	+1.08	+3.06	−1.87
303.2	255.7	323.9	0.789	−0.20	+1.16	−1.37
66.9	55.4	69.2	0.801	−1.95	−2.12	+0.13

Diethylene glycol was used in a similar test of the apparatus since none is lost by volatilization; the results obtained are given in Table II.

The errors observed are insignificant in the calculation of physiological data.

SUMMARY

A non-ventilated closed chamber is described for the measurement of the respiratory exchange of the rat. By maintaining the chamber at atmospheric pressure the oxygen consumption may be calculated from an analysis for carbon dioxide and the change in the volume of the system. No time-consuming temperature equilibration of the system is required. With this simple apparatus one operator may perform serial measurements of short duration of the respiratory exchange.

MICROBIOLOGICAL DETERMINATION OF ARGININE IN PROTEINS AND FOODS

By MILLARD J. HORN, D. BREESE JONES, AND AMOS E. BLUM

(From the Bureau of Human Nutrition and Home Economics, Agricultural Research Administration, United States Department of Agriculture, Washington)

(Received for publication, May 12, 1948)

Lactobacillus casei (1–4), *Lactobacillus arabinosus* (5), *Leuconostoc mesenteroides* P-60 (6–7), *Lactobacillus fermenti* 36 (8), and *Streptococcus faecalis* (9–14) have all been proposed or used for the assay of arginine. The assays reported in this paper were made with *Streptococcus faecalis* and with the medium previously used for threonine, valine, and histidine (15–17).

EXPERIMENTAL

Streptococcus faecalis 9790[1] was employed in the assays described.

Basal Medium—The basal medium was the same as that described in a previous paper for methionine (18), with the exception that pyridoxine was replaced by 400 γ of pyridoxamine.

Assay Procedure—The procedures followed for the cultures, inoculum, and preparation of samples were identical with those described in other papers (15, 18).

Preparation of Arginine Standards—Suitable concentrations of L-arginine monohydrochloride were used for the development of the standard curve. The titration values on the standard curve (Fig. 1) were not altered by the addition to the medium of 1.2 mg. of any of the other nineteen amino acids.

The recovery of arginine added to hydrolysates of casein, whole corn, rye, and zein gave results well within the experimental error for this type of assay (Table I).

Table II shows values found for several foods and proteins at different assay levels. Data on the reproducibility of values found for nine materials of diverse character are shown in Table III.

The results (Table IV) found for the proteins and foods[2] agree well with other microbiological methods.

The values reported by Vickery (19) on six proteins and those of Bergdoll and Doty (21) on four proteins are in close agreement with our results.

---

[1] Obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D. C.

[2] The sources and preparation of the samples assayed are given in a previous publication on the determination of methionine (31).

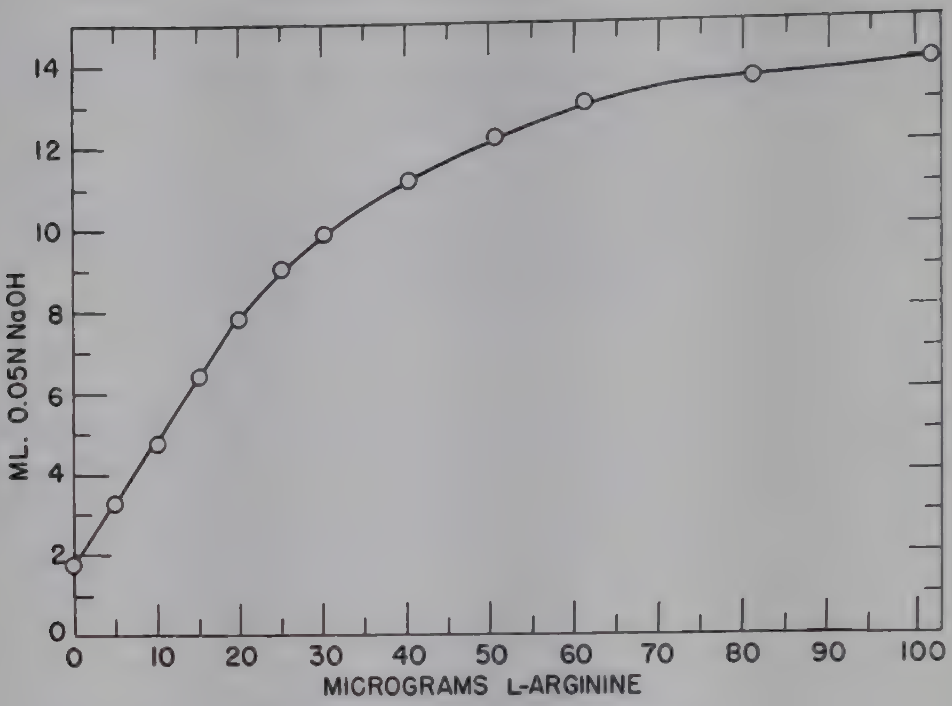


FIG. 1. Standard titration curve

TABLE I

Recovery of Arginine Added to Protein Hydrolysates

Protein hydrolysate	Arginine				
	In hydrolysate*	Added	Total	Found	Recovery
	γ	γ	γ	γ	per cent
Casein	3.50	20	23.50	23.50	100
	7.00	20	27.00	27.60	102
	10.50	20	30.50	30.70	101
	14.00	20	34.00	34.00	100
Corn, whole, yellow	5.50	5	10.50	10.00	95
	5.50	10	15.50	15.30	98
	5.50	15	20.50	20.80	101
	5.50	20	25.50	25.70	101
Rye, whole	5.90	10	15.90	15.80	99
	11.80	10	21.80	22.00	101
	17.70	10	27.70	27.70	100
Zein	3.50	5	8.50	8.00	94
	3.50	10	13.50	13.50	100
	3.50	15	18.50	18.50	100
	3.50	20	23.50	24.00	102

* Not corrected for moisture and ash.

Vickery used flavianic acid and Bergdoll and Doty a chromatographic separation in assaying this amino acid.

TABLE II

*Arginine Content at Different Assay Levels**

Material	Level added	Found	Material	Level added	Found
	γ	*per cent*		γ	*per cent*
Arachin	100	12.5	Soy bean flour	200	3.85
	200	12.5		400	4.00
	300	12.1		600	3.70
	400	12.5		800	3.85
Average............		12.4			3.85
Milk, dry, skim	200	0.95	Wheat bran globulin	100	12.70
	400	0.95		200	12.75
	600	0.95		300	12.67
	800	0.96		400	12.75
Average............		0.95			12.72
Oatmeal	500	1.10	Wheat germ, defatted	200	2.50
	1000	1.15		400	2.43
	1500	1.13		600	2.50
	2000	1.14		800	2.48
Average............		1.13			2.48
Rye, whole	1000	0.60			
	2000	0.60			
	3000	0.60			
	4000	0.60			
Average............		0.60			

* Not corrected for moisture and ash.

TABLE III

*Reproducibility (Per Cent) of Arginine Content When Determined by Separate Assays**

Material	Assay 1	Assay 2	Average
Casein........................	3.50	3.56	3.53
Conarachin....................	15.61	15.37	15.49
Corn, whole, yellow............	0.54	0.55	0.55
Cottonseed flour...............	6.67	6.85	6.76
Egg, whole, dried..............	4.28	4.39	4.33
Milk, dry, skim................	1.01	0.96	0.99
Peanut, total globulins........	13.25	13.36	13.30
Rye, whole.....................	0.60	0.58	0.59
Zein..........................	1.82	1.90	1.86

* Not corrected for moisture and ash.

SUMMARY

A microbiological method is described for the determination of arginine in proteins and foods with *Streptococcus faecalis*. The results of assays on thirty-one proteins and foods agree closely with those obtained on the same materials by both chemical and microbiological methods.

TABLE IV

Arginine Content of Some Proteins and Foods

Percentages calculated for ash- and moisture-free material.

Material	N	Arginine	Values from literature
	per cent	*per cent of food*	
Arachin	18.30	13.50	13.94 (19), 15.4 (20)
Casein	16.07	3.81	3.7 (2), 3.6 (4), 3.7 (7), 3.9 (10), 3.6 (11), 3.72 (19), 3.7 (21), 4.3 (20), 3.60 (22)
Coconut globulin	17.42	16.73	15.92 (23)
Conarachin	18.20	16.53	12.7 (20)
Cottonseed globulin	18.00	14.72	14.92 (19), 13.6 (24)
Edestin	18.55	16.51	16.7 (13), 16.76 (19), 14.0 (20)
Gelatin (Bacto)	18.32	9.11	9.30 (2), 8.0 (4), 9.1 (10), 8.68 (19), 9.08 (21)
Glycinin	17.30	7.94	8.5 (20)
Lactalbumin	15.39	3.42	3.2 (4), 3.4 (21)
Ovalbumin (crystalline)	15.98	6.03	6.6 (2), 5.7 (7), 5.94 (10), 5.66 (19), 6.0 (21)
Ox muscle	16.00	7.87	6.0 (4), 6.3 (20), 6.9 (25)
Peanut, total globulins	18.01	14.16	
Phaseolin (navy bean)	16.07	5.97	
Wheat bran globulin	17.76	13.30	
Zein	16.00	1.95	1.60 (19), 1.80 (20)
Barley, pearled	1.86	0.53	0.32 (14)
Brazil nut meal	9.03	7.62	
Corn germ, defatted	3.93	2.18	1.67 (20), 2.48 (26)
" whole, yellow	2.22	0.65	0.59 (14), 0.56 (20), 0.46 (27)
Cottonseed flour	10.36	7.72	4.87 (14), 4.79 (20), 8.55 (26)
Egg, whole, dried	8.11	4.82	3.24 (28)
Milk, dry, skim	6.57	1.15	1.48 (10), 1.31 (11), 1.24 (14)
Oatmeal	2.73	1.26	0.70 (14), 1.02 (20)
Peanut flour	10.15	7.76	6.29 (20), 8.75 (26)
Peas, black-eyed	4.15	1.95	
Rice, white	1.26	0.69	0.57 (20)
Rye, whole	1.98	0.67	0.53 (10)
Soy bean flour	8.85	4.33	3.93 (10), 2.92 (14), 4.15 (29)
Wheat germ, defatted	6.50	2.80	2.44 (20), 3.90 (26)
" whole	3.07	0.86	0.81 (10), 0.59 (14), 0.63 (20)
Yeast, dried, brewers'	7.71	2.26	2.17 (10), 2.08 (30)

BIBLIOGRAPHY

1. Hutchings, B. L., and Peterson, W. H., *Proc. Soc. Exp. Biol. and Med.*, **52,** 36 (1943).
2. McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, **152,** 83 (1944).
3. Shankman, S., Dunn, M. S., and Rubin, L. B., *J. Biol. Chem.*, **151,** 511 (1943).
4. Hier, S. W., Graham, C. E., Freides, R., and Klein, D., *J. Biol. Chem.*, **161,** 705 (1945).
5. Hegsted, D. M., *J. Biol. Chem.*, **152,** 193 (1944).
6. Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, **156,** 703 (1944).
7. Guirard, B. M., Snell, E. E., and Williams, R. J., *Proc. Soc. Exp. Biol. and Med.* **61,** 158 (1946).
8. Dunn, M. S., Camien, M. N., and Shankman, S., *J. Biol. Chem.*, **161,** 657 (1945).
9. Snell, E. E., and Guirard, B. M., *Proc. Nat. Acad. Sc.*, **29,** 66 (1943).
10. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, **160,** 35 (1945).
11. Hodson, A. Z., and Krueger, G. M., *Arch. Biochem.*, **10,** 55 (1946).
12. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, **166,** 417 (1946).
13. Smith, E. L., and Greene, R. D., *J. Biol. Chem.*, **167,** 833 (1947).
14. Baumgarten, W., Mather, A. N., and Stone, L., *Cereal Chem.*, **23,** 135 (1946).
15. Horn, M. J., Jones, D. B., and Blum, A. E., *J. Biol. Chem.*, **169,** 739 (1947).
16. Horn, M. J., Jones, D. B., and Blum, A. E., *J. Biol. Chem.*, **170,** 719 (1947).
17. Horn, M. J., Jones, D. B., and Blum, A. E., *J. Biol. Chem.*, **172,** 149 (1948).
18. Horn, M. J., Jones, D. B., and Blum, A. E., *J. Biol. Chem.*, **166,** 321 (1946).
19. Vickery, H. B., *J. Biol. Chem.*, **132,** 325 (1940).
20. Block, R. J., and Bolling, D., The amino acid composition of proteins and foods, Springfield (1945).
21. Bergdoll, M. S., and Doty, D. M., *Ind. and Eng. Chem., Anal. Ed.*, **18,** 600 (1946).
22. Albanese, A. A., and Frankston, J. E., *J. Biol. Chem.*, **159,** 185 (1945).
23. Jones, D. B., and Johns, C. O., *J. Biol. Chem.*, **44,** 291 (1920).
24. Fontaine, T. D., Olcott, H. S., and Lowy, A., *Ind. and Eng. Chem.*, **34,** 116 (1942).
25. Beach, E. F., Munks, B., and Robinson, A., *J. Biol. Chem.*, **148,** 431 (1943).
26. Edwards, L. E., Sealock, R. R., O'Donnell, W. W., Bartlett, G. R., Barclay, M. B., Tully, R., Tybout, R. H., Box, J., and Murlin, J. R., *J. Nutr.*, **32,** 597 (1946).
27. Doty, D. M., Bergdoll, M. S., Nash, H. A., and Brunson, A. M., *Cereal Chem.*, **23,** 199 (1946).
28. Mitchell, H. H., and Block, R. J., *J. Biol. Chem.*, **163,** 599 (1946).
29. Riesen, W. H., Clandinin, D. R., Elvehjem, C. A., and Cravens, W. W., *J. Biol. Chem.*, **167,** 143 (1947).
30. Block, R. J., and Bolling, D., *Arch. Biochem.*, **7,** 313 (1945).
31. Horn, M. J., Jones, D. B., and Blum, A. E., *J. Biol. Chem.*, **166,** 313 (1946).

THE AVAILABILITY OF DL-LANTHIONINE FOR THE PROMOTION OF GROWTH IN YOUNG RATS WHEN ADDED TO A CYSTINE- AND METHIONINE-DEFICIENT DIET

By D. BREESE JONES, ALVIN CALDWELL, and MILLARD J. HORN

(From the Bureau of Human Nutrition and Home Economics, Agricultural Research Administration, United States Department of Agriculture, Washington)

(Received for publication, May 12, 1948)

In an earlier communication (1) it was reported that the internally compensated thio ether amino acid, mesolanthionine, is not utilized for the growth of young rats subsisting on a cystine- and methionine-deficient diet. Two alternative explanations were given for this failure of the animals to utilize mesolanthionine, either that the animals were unable to split the thio ether or that cleavage did occur in such a way that the unutilizable D-cysteine (2) was formed instead of L-cysteine. In order to develop information on this question, feeding experiments have now been conducted with DL-lanthionine. By feeding the racemic form under the same conditions by which the mesolanthionine was fed, it was believed that evidence could be obtained to show which of the above alternative explanations for the failure of mesolanthionine to support growth is correct. If the L component of the DL-lanthionine were split, one-fourth of the DL-lanthionine would supply L-cysteine irrespective of what side of the sulfur in the molecule cleavage occurred. The results of the experiments here reported show that DL-lanthionine supports growth to the extent that the L component is cleaved to yield 1 molecule of L-cysteine.

DL-Lanthionine usually accompanies mesolanthionine in acid hydrolysates of alkali-treated proteins (3). The DL-lanthionine used in these experiments was obtained from acid hydrolysates of human hair that had been previously boiled for 1 hour with 2 per cent Na_2CO_3 solution.[1]

EXPERIMENTAL

In the previous investigation (1) on the nutritional availability of mesolanthionine, to supply the B vitamins a cystine and methionine basal diet having the following percentage composition was satisfactorily used: casein 6.0, dextrin 37.0, sucrose 15, salt mixture 4 (4), agar 2.0, lard 19, cod liver oil 5.0, and a commercial milk-vitamin concentrate 12. When fed this diet the animals invariably lost weight.

---

[1] Tests made on the DL-lanthionine for disulfide gave negative results, showing that it was free from any trace of cystine.

Since the milk-vitamin concentrate previously used was no longer available, a simpler basal diet containing synthetic vitamins and choline was first employed. It was found, however, that with 6 per cent casein in the diet, as previously used with the milk-vitamin concentrate, the animals grew too fast to make it possible to detect the effect of adding the amino acid supplements. By omitting choline and reducing the level of casein to 4.21 per cent, a satisfactory basal diet was obtained. When fed this diet, the animals declined rapidly in weight. Addition of the sulfur amino acids promptly arrested the decline in weight, and growth proceeded at a satisfactory rate. Salmon (5) has pointed out that the primary deficiency of casein at low levels is in labile methyl groups. This is in accord with the decline in weight of our rats when choline was omitted from the diet. However, this diet with a low content of fat, devoid of choline and containing 4.21 per cent of casein, supported growth at a satisfactory rate after it had been supplemented with cystine as well as with choline, methionine, or DL-lanthionine. Unless the cod liver oil or corn oil used in the diet contained an appreciable amount of choline, the methionine in the casein may have supplied enough methyl groups to be adequate for growth when cystine was added.

The data presented represent the results obtained with eleven lots of albino rats. In addition to these, several lots were used in preliminary work on developing a satisfactory basal diet. Each lot consisted of four or six animals from the same litter, equally divided with respect to sex and having initial weights of 50 to 60 gm. They were housed in individual cages having wide mesh screen bottoms and kept in a room maintained at about 24.4°. The animals were weighed twice weekly. After considerable experimentation a basal diet having the following percentage composition was found satisfactory: casein (Labco) 4.21, dextrinized corn-starch 90.79, salt mixture 2.00 (4), cod liver oil 2.00, and corn oil 1.00. A vitamin mixture was incorporated in the dextrinized corn-starch which provided the following constituents in each 100 gm. of diet: thiamine hydrochloride 0.2 mg., pyridoxine hydrochloride 0.2 mg., riboflavin 0.3 mg., calcium pantothenate 0.3 mg., and niacin 1 mg. These vitamins, in an aqueous-alcoholic solution, were added to the dextrinized starch and the mixture was dried at 50° to the original weight of the starch. The diet was fed *ad libitum* and a record of the food intake is given in Table I. It is of interest to note that in general the food consumption was definitely lower during the periods when the animals subsisted on the basal diet alone than when the amino acid supplements were incorporated.

The animals fed the basal diet alone invariably lost weight rapidly, and when the feeding was prolonged they died in about 45 days. For comparison with the behavior of the rats given DL-lanthionine, L-cystine and

DL-methionine were also used. The addition of 0.3 gm. of cystine or 0.37 gm. of methionine to 100 gm. of the basal diet made it effective in promptly arresting the decline in weight of the animals, and in enabling them to grow at a satisfactory rate. Likewise, equally prompt and effective responses followed the addition of 1.04 gm. of DL-lanthionine to the basal diet. The above-stated amounts of the three supplements added to the diet represent biologically available sulfur equivalents.

TABLE I*

Food Consumption

Lot No.	Rat No.	Experimental period	Supplement to basal diet	Average daily food consumption
		days	*per cent*	*gm.*
210	4 ♀	41	0.30 cystine	11.9
213	1 ♂	23	No supplement	6.6
		47	0.37 methionine	6.8
208	2 ♀	22	No supplement	4.9
		26	0.30 cystine	6.4
218	2 ♀	20	No supplement	5.7
		14	1.04 DL-lanthionine	5.6
		17	No supplement	2.1
220	2 ♂	11	1.04 DL-lanthionine	9.4
		8	No supplement	3.1
		6	1.04 DL-lanthionine	4.7
220	3 ♀	11	1.04 “	10.3
		8	No supplement	3.4
		6	1.04 DL-lanthionine	5.5
211	1 ♂	40	0.37 methionine	9.3
212	4 ♀	24	No supplement	6.2
		16	0.30 cystine	7.0
		16	No supplement	6.3
		9	0.30 cystine	10.6
220	1 ♀	12	1.04 DL-lanthionine	8.4
		7	No supplement	4.4
		7	1.04 DL-lanthionine	4.7
218	4 ♀	18	No supplement	5.9
		14	1.04 DL-lanthionine	6.6
		18	No supplement	4.0
218	3 ♂	20	“ “	5.1
		13	1.04 DL-lanthionine	4.4
		16	No supplement	3.9
218	1 ♂	42	“ “	3.2

* This table includes the record of only those rats, the growth curves of which are shown in Figs. 1 and 2.

In six of the lots all of the animals were fed the basal diet from the start for periods of 15 to 25 days. Following the fore periods on the basal diet the animals were fed one of the supplements. In most cases, one rat of a lot was allowed to continue on the basal diet throughout the remainder of the feeding period to serve as a control. In several lots the effect of the supplements was studied throughout successive periods on the same animal.

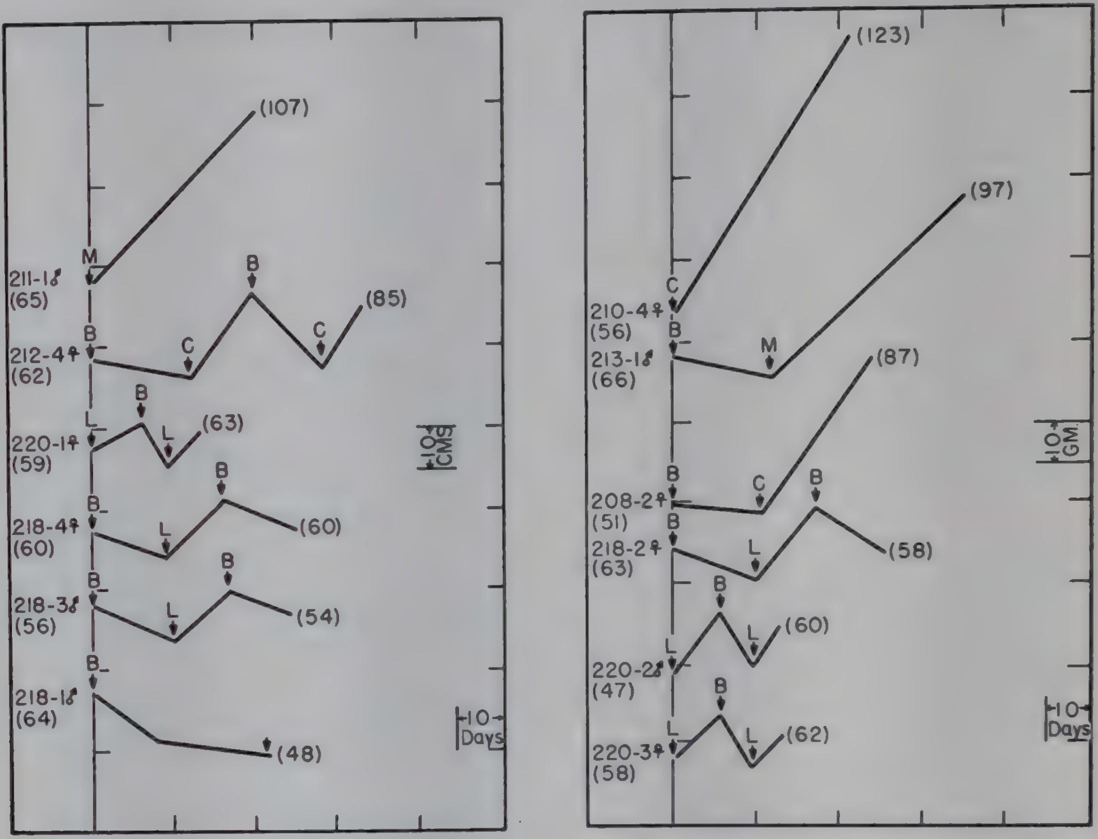


Fig. 1

Fig. 2

Figs. 1 and 2. Growth curves of rats receiving the basal-deficient diet (Diet B) alone, or supplemented with 0.3 per cent of L-cystine (Diet C), 0.37 per cent of DL-methionine (Diet M), or with 1.04 per cent of DL-lanthionine (Diet L). The introduction of each diet is indicated by the symbols over the downward arrows. The identification number and sex of the rats are given on the extreme left with their initial and final weights in parentheses.

Without exception, DL-lanthionine proved to be as effective as cystine or methionine in its capacity to promote growth at the start, or to restore it after a period of decline on the basal diet alone. The growth curves for representative animals from the various lots are given in Figs. 1 and 2. These are typical of the others which are omitted to save space.

SUMMARY

Feeding experiments have shown that racemic lanthionine can replace cystine and methionine in the diet. Young albino rats fed a low protein (casein) basal diet deficient in cystine, but adequately supplied with the non-protein dietary essential factors, declined in weight rapidly. For comparison with the behavior of rats given racemic lanthionine, L-cystine and DL-methionine were also fed. The addition of 0.3 gm. of L-cystine or 0.37 gm. of DL-methionine per 100 gm. of basal diet caused immediate resumption of growth. The addition of DL-lanthionine likewise resulted in corresponding growth resumption. An immediate decline occurred when the lanthionine was omitted from the basal diet, and growth again was resumed when the lanthionine was supplied. Previous experiments similarly conducted with mesolanthionine showed that this isomer is not utilized for growth.

BIBLIOGRAPHY

1. Jones, D. B., Divine, J. P., and Horn, M. J., *J. Biol. Chem.*, **146**, 571 (1942).
2. du Vigneaud, V., Dorfman, R., and Loring, H. S., *J. Biol. Chem.*, **98**, 577 (1932).
3. Horn, M. J., Jones, D. B., and Ringel, S. J., *J. Biol. Chem.*, **144**, 93 (1942).
4. Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutr.*, **14**, 273 (1937).
5. Salmon, W. D., *J. Nutr.*, **33**, 155 (1947).

A NEW SYNTHESIS OF CYSTINE*

By M. W. FARLOW

(From the Chemical Department, Experimental Station, E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware)

(Received for publication, June 21, 1948)

The present paper describes a new and convenient synthesis of cystine from pyruvic acid. The essential steps are illustrated in the following reaction scheme:

$$CH_3COCO_2H + CH_3CONH_2 \rightarrow CH_2 = C(NHCOCH_3)CO_2H \qquad (A)$$

$$(A) + CH_3COSH \rightarrow CH_2(SCOCH_3)CH(NHCOCH_3)CO_2H \qquad (B)$$

$$(B) + H_2O + (O) \rightarrow (\text{—}SCH_2CHNH_2CO_2H)_2$$

Acetamidoacrylic acid is prepared from pyruvic acid and acetamide by the method of Bergmann and Grafe (2). As pointed out by previous investigators (2–4), α-acetamidoacrylic acid, an eneamide, can also have a tautomeric imide form, but the amide structure is believed to express better the behavior of the compound in the present cystine synthesis.

Addition of thiolacetic acid to α-acetamidoacrylic acid is accomplished in the presence of a trace of ascaridole as catalyst, giving N,S-diacetyl-DL-cysteine, m.p. 118°, in a yield of 85 per cent of the theoretical. N,S-Diacetylcysteine of unspecified optical character has been prepared previously by the action of ketene on cysteine and reported to melt at 111–112° (5) and at 109–110° (6). Hydrolysis of the diacetyl derivative with concurrent oxidation by iodine gives 72 per cent of the theoretical amount of cystine.

This paper is based on work carried out under the supervision of Dr. W. A. Lazier[1] for the Office of Scientific Research and Development under Contract OEMsr-377 with E. I. du Pont de Nemours and Company.

EXPERIMENTAL

α-Acetamidoacrylic Acid—α-Acetamidoacrylic acid and α,α-bis(acetamido)propionic acid were obtained by heating pyruvic acid with acetamide, as described by Bergmann and Grafe (2). Warming the bisacetamido compound with acetic acid produced additional amounts of the desired acrylic derivative, as reported by these authors.

N,S-Diacetyl-DL-cysteine—To a mixture of 1.78 gm. of α-acetamidoacrylic acid and 10 ml. of thiolacetic acid (7) was added a fraction of a drop of ascaridole, and the mixture was refluxed for 20 minutes until solution was complete, and then for an additional 15 minutes. Evaporation of the reaction mixture in a vacuum desiccator gave a crystalline residue which was recrystallized from a mixture of chloroform and petroleum ether. The recrystallized addition product melted at 118° and had a neutral equivalent of 203 as compared with the theoretical value of 205 for diacetylcysteine. The yield was 2.41 gm. (85 per cent).

---

* Contribution No. 237; see Farlow (1).

[1] Present address, Chas. Pfizer and Company, Inc., Brooklyn, New York.

Cystine—A solution of 1.03 gm. of diacetylcysteine in 10 ml. of concentrated hydrochloric acid was boiled gently and a 0.53 N solution of iodine in methanol was added from a burette in portions of about 0.2 ml., as rapidly as decolorized until a permanent color of iodine remained for 5 minutes after the last addition. 10 ml. of iodine solution, or about 105 per cent of the theoretical quantity, were required. The addition covered a period of 30 minutes. The solution was evaporated almost to dryness, diluted with water, and sodium acetate was added until the solution was alkaline to Congo red paper. The cystine, which separated very slowly, was removed by filtration and washed with water, alcohol, and ether. The dry product weighed 0.43 gm. or 72 per cent of the theoretical.

Analysis—$C_6H_{12}N_2O_4S_2$. Calculated. C 30.4, H 5.4, N 11.0, S 26.4
Found. " 30.0, " 5.0, " 11.7, " 26.6

As further confirmation of its identity, the cystine was converted by sodium-ammonia reduction and alkylation into S-benzyl-DL-cysteine, and the latter by acetylation into S-benzyl-N-acetyl-DL-cysteine by the methods of du Vigneaud and coworkers (8). These derivatives were found to melt at 215–216° and 157°, respectively, as compared with the values of 215–216° and 158° reported by du Vigneaud.

SUMMARY

A new synthesis of cystine is presented. Pyruvic acid is treated with acetamide to give α-acetamidoacrylic acid, to which is added thiolacetic acid to produce N,S-diacetyl-DL-cysteine. Hydrolysis and oxidation of the latter produce cystine.

BIBLIOGRAPHY

1. Farlow, M. W., U. S. patent 2,406, 362 (1946).
2. Bergmann, M., and Grafe, K., *Z. physiol. Chem.*, **187**, 187 (1930).
3. Shemin, D., and Herbst, R. M., *J. Am. Chem. Soc.*, **60**, 1954 (1938).
4. Bergmann, M., Miekeley, A., and Kann, E., *Z. physiol. Chem.*, **146**, 247 (1925).
5. Neuberger, A., *Biochem. J.*, **32**, 1452 (1938).
6. Perez, J. J., and Sandor, G., *Bull. soc. chim. Biol.*, **22**, 149 (1940).
7. Ellingboe, E. K., U. S. patent 2,412,036 (1946).
8. du Vigneaud, V., Audrieth, L. F., and Loring, H. S., *J. Am. Chem. Soc.*, **52**, 4500 (1930).

A COLORIMETRIC METHOD FOR ESTIMATION OF DEHYDROISOANDROSTERONE AND ITS APPLICATION TO URINE EXTRACTS*

By PAUL L. MUNSON, MARY ELLEN JONES, PHILIP J. McCALL, and T. F. GALLAGHER†

(*From the Department of Biochemistry, University of Chicago, and the Endocrinology Research Section, Armour and Company, Chicago*)

(Received for publication, May 19, 1948)

Kerr and Hoehn (2), while investigating the specificity of a modified Pettenkofer reaction, observed that dehydroisoandrosterone (DHA) gave a strongly positive reaction. The similar behavior of cholic acid had long been known, and a number of procedures for its quantitative estimation based on this color reaction have been described (3–5). We have found that the Schmidt and Hughes procedure (4) for the determination of cholic acid can be adapted to the photoelectric colorimeter and used for the quantitative determination of DHA. Further studies on the specificity of the reaction for DHA, on a quantitative basis, have enabled us to make certain generalizations concerning the structural features necessary for a positive color test. We have also made a preliminary investigation of the application of the color reaction to extracts of human urine and, with certain reservations which will be discussed, we believe that the color reaction provides a simple analytical method for DHA applicable to ex-extracts from natural sources. The method should be of value in the further investigation of the metabolism of DHA in normal and pathological individuals, particularly the rôle it may play as an intermediate in the metabolism of the adrenal cortical hormones.

EXPERIMENTAL

Reagents—

1. *Furfural solution.* A commercial grade of furfural is distilled twice on a boiling water bath under reduced pressure. The middle fraction only is retained from each distillation. For a satisfactory product it is essential

---

* Preliminary reports were presented at the American Association for the Advancement of Science Gibson Island conference on hormones, 1943 (1), and at the Technological Conference, Chicago Section of the American Chemical Society, January 24, 1947.

This work was supported in part by a grant from Armour and Company and in part by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

† Present address, Sloan-Kettering Institute for Cancer Research, New York.

that the temperature of a boiling water bath is not exceeded. The nearly colorless product is promptly dissolved in 50 per cent acetic acid at a concentration of 0.56 per cent (volume per volume) and stored in the cold. We have found the reagent, stored at −5°, to be stable for many months.

2. *DHA standard solutions.* A solution containing 10 mg. of DHA or 11.45 mg. of DHA acetate per 100 ml. of glacial acetic acid is prepared, and additional standards containing 0.060 and 0.020 mg. of DHA per ml. are obtained by dilution with glacial acetic acid. In those experiments in which DHA was used as a standard, the substance melted at 139–141° (corrected); $[\alpha]_D^{21} = +10.4°$ (ethanol). Because DHA crystallizes in two polymorphic modifications, the melting point is an unsatisfactory criterion of purity. We have therefore, for the greater portion of the work, used the acetate of this compound (m.p. 169–170.5° (corrected); $[\alpha]_D = +4.2°$ (ethanol)) as the standard but have expressed the results in terms of DHA. The acetate gives the same intensity of color mole for mole as the free hydroxy ketone.

3. *Sulfuric acid, 16.0 N.*

4. *Acetic acid, 50 per cent by weight.*

Provided all test solutions are treated uniformly, minor changes in reagent concentrations, temperature, and time are relatively unimportant. The recommended concentrations of reagents differ from those of Schmidt and Hughes (4) in preparation; their use leads to the same concentrations in the final mixture as specified by these authors.

An amount of pure steroid or of urine extract estimated to contain from 10 to 50 γ of DHA or its equivalent is transferred to a calibrated Evelyn colorimeter tube and evaporated to dryness on a water bath under a stream of nitrogen. The dry residue, if the evaporation has been properly carried out, is confined to a small area in the bottom of the tube and is dissolved in 0.5 ml. of glacial acetic acid, with warming if necessary. The solutions in Table I are then added to Evelyn tubes in duplicate or triplicate and mixed.

To each tube indicated in Table I, 7.5 ml. of 16 N sulfuric acid are added at 1 minute intervals. After mixing well, the tube is placed in an efficient, large capacity, constant temperature water bath maintained at 67 ± 0.2°. After exactly 12 minutes in the bath, the tube is removed and immediately placed in an ice bath for 1 minute. After all the tubes have been heated and cooled (a series of thirty to forty tubes can be analyzed conveniently), the color intensity is determined in the Evelyn colorimeter, with Filter 660, the wave-length of maximum absorption of the colored product. A center setting is obtained by adjusting the "blank without furfural" to 100. Since all the solutions containing furfural increase slightly in color while standing at room temperature, the colorimetric measurements are made at approximately the same time interval (±10 minutes) after removal from the bath.

There is a small but significant day to day variation in the color intensity developed by DHA standards, even though the same reagents are used and the assay conditions are apparently identical. It is therefore essential that a full set of standards be included in each assay series. The color produced is affected significantly by changes in furfural concentration, H_2SO_4 concentration, bath temperature, and heating time. Therefore it is also essential to use the same reagents and assay conditions for standards, blanks, and extracts.

TABLE I

Reagents for Dehydroisoandrosterone Determination

	Glacial acetic acid	DHA standard solution	50 per cent acetic acid	Furfural solution	Urine extract in glacial acetic acid
	ml.	*ml.*	*ml.*	*ml.*	*ml.*
Blank without furfural	0.5		2.0		
Reagent blank	0.5			2.0	
DHA standards (10, 30, and 50 γ levels)		0.5		2.0	
Urine extract blank			2.0		0.5
" "				2.0	0.5

Calculations

1. The galvanometer readings (G) are converted to L values. ($L = 2 - \log G$. A convenient table for conversion is included in the manual accompanying the Evelyn colorimeter.) If replicate determinations have been made, the mean L value for each set of replicates is calculated.

2. To eliminate non-specific color (a) originally present in the extract and (b) produced by the action of H_2SO_4 alone on the extract, the L value for the "urine extract blank" is subtracted from that of the "urine extract." Occasional urine extracts develop a slight turbidity, which also is corrected by the urine extract blank. The mean L values of the reagent blank and the DHA standards are plotted on graph paper and a curve drawn connecting the points. (The curve deviates slightly from strict linearity, thus differing from that obtained in the analysis of cholic acid.) The DHA content of the urine extract aliquot is estimated by interpolation on the graph, and the total DHA content of the extract is obtained by application of the appropriate factor. An example of a standard curve is shown in Fig. 1, and a sample set of calculations is given in Table II.

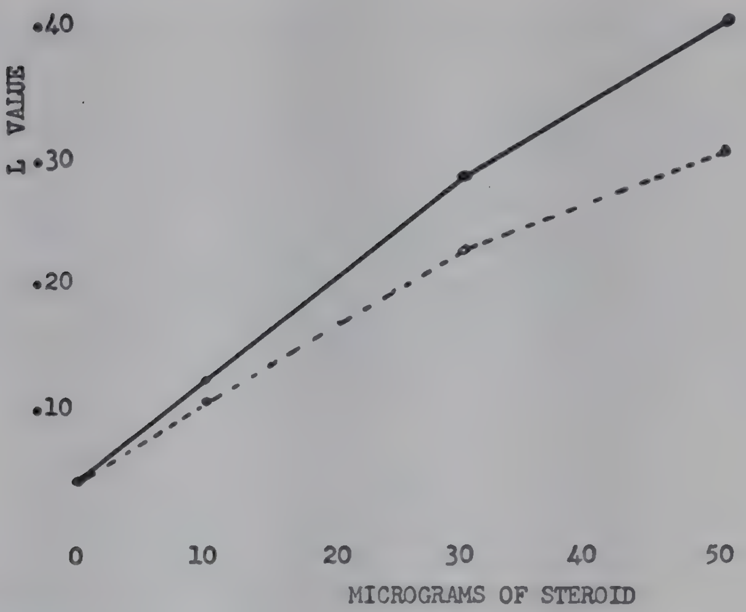


FIG. 1. Calculation of standard curve. $L = 2 - \log G$. The solid curve represents dehydroisoandrosterone or 3(β)-chloroandrostenone-17; the dotted curve $\Delta^{3,5}$-androstadienone-17.

TABLE II

Method of Calculation of Results in DHA Colorimetric Assay

Determination	Mean L value	Estimated DHA content in aliquot	Factor	Total DHA content
				mg.
No furfural blank	0.0000			
Reagent blank	0.0459			
10 γ DHA standard	0.1257			
30 " " "	0.2818			
50 " " "	0.4034			
Urine Extract 57-16 (total volume, 50 ml.) 0.4 ml. 1:5 dilution				
Extract	0.3714			
Blank	0.0163			
Net	0.3551	42.2	625	26.4
0.2 ml. 1:5 dilution				
Extract	0.2231			
Blank	0.0078			
Net	0.2153	21.5	1250	26.8
Mean value for Extract 57-16				26.6

Results

Table III demonstrates the reliability of the colorimetric method when it is applied to "unknown" solutions of DHA, to mixtures of DHA and androsterone, and to a crude urine extract with and without added DHA. It is apparent from these results that DHA can be assayed in the presence of a non-chromogenic substance and that added DHA can be quantitatively measured in the presence of a relatively crude urine extract. Since the results suggested that the colorimetric determination of DHA in urine extracts was possible, the specificity of the reaction was investigated. Table IV lists a series of neutral steroids tested which gave positive results when the color reaction was applied under the conditions previously

TABLE III

Application of Method to Unknown Solutions

Solution No.	DHA added	Androsterone added	Urine Extract 409 added	DHA found	Per cent recovery
	γ	γ	*ml.*	γ	
1	32.5			33.0	101.5
2	22.5			22.5	100
3	32.0			32.4	101.2
4	12.0			12.0	100
5	20.0	40		20.2	101
6	40.0	80		39.8	99.5
7	50.0	100		50.0	100
8	0		0.5	14.5	
9	10.0		0.5	24.5	100
10	0		1.0	29.0	
11	20.0		1.0	49.0	100

TABLE IV

Color Intensity from Application of Method to Pure Compounds

	per cent
Dehydroisoandrosterone	100
" acetate	100
Potassium dehydroisoandrosterone sulfate	105
Δ^5-3(β)-Chloroandrostenone-17	102
$\Delta^{3,5}$-Androstadienone-17	80
Δ^5-Pregnenol-3(β)-one-20	78
Δ^5-Pregnenediol-3(β)21-one-20,21-acetate	>100
Δ^5-17-Ethinylandrostenediol-3(β),17(β)	75
Δ^5-17-Methylandrostenediol-3(β),17(β)	25
Δ^5-Androstenediol-3(β),17(α)	25
Δ^5-Androstenediol-3(β),17(β)	25
Δ^5-Androstenetriol-3(β),16,17	75
Δ^5-Androstenol-3(β)	55
$\Delta^{3,5}$-Androstadienol-17-acetate	25
Δ^{16}-Allopregnenediol-3(α),20(β)	25
Δ^5-Pregnenediol-3(β),20(β)	<20

TABLE V

Pure Compounds Tested with Negative Results at 650 mμ

C_{19}-saturated	Androstanol-3(α)-one-17 (androsterone)
	Etiocholanol-3(α)-one-17
	Androstanol-3(β)-one-17 (isoandrosterone)
	Etiocholanol-3(α)-dione-11,17
	Androstanediol-3(α),11(β)-one-17
	Androstanol-5-trione-3,6,17
	Androstanedione-3,17
	Etiocholanetrione-3,11,17
	Androstanediol-3(α),17(α)
	Androstanetriol-2,3,17
C_{19}-unsaturated	$\Delta^{2\text{ or }3}$-Androstenone-17
	Δ^{5}-Androstenol-3(α)-one-17
	Δ^{9}-Etiocholenol-3(α)-one-17
	Δ^{4}-Androstenol-17(α)-one-3 (testosterone)
	Δ^{4}-Androstenedione-3,17
	Δ^{5}-Androstenedione-3,17
	Δ^{4}-Androstenetrione-3,11,17 (adrenosterone)
	Δ^{4}-Androstenetriol-3(β),4,17(α)
	Δ^{4}-17-Methylandrostenol-17(β)-one-3 (methyl testosterone)
C_{21}-saturated	Pregnanol-3(α)-one-20
	Pregnanol-20(α)-one-3
	Allopregnanol-3(α)-one-20
	Allopregnanol-3(β)-one-20
	Pregnanedione-3,20
	Allopregnanedione-3,20
	Pregnanetrione-3,11,20
	Allopregnanetrione-3,6,20
	Allopregnanediol-3(β),6(α)-one-20
	Pregnanediol-3,4-one-20-diacetate
	Pregnanediol-3(α),17(α)-one-20
C_{21}-unsaturated	Δ^{4}-Pregnenedione-3,20 (progesterone)
	Δ^{4}-Pregnenol-17(α)-dione-3,20
	Δ^{4}-17-Ethinylandrostenone-3
	$\Delta^{5,16}$-Pregnadiendiol-3,4-one-20-diacetate
	Δ^{16}-Allopregnenediol-3(β),6(β)-one-20
	Δ^{16}-Pregnenedione-3,20
	17-Isopropylidenetiocholanol-3(β)
	Δ^{4}-Pregnenediol-11(β),21-dione-3,20 (corticosterone)
	Δ^{4}-Pregnenediol-17(α),21-trione-3,11,20 (Kendall's Compound E)
	Δ^{4}-Pregnenol-21-dione-3,20 (desoxycorticosterone)
Phenols	Estrone
	α-Estradiol
	Estriol

specified. In Table V is listed a series of neutral steroids tested which gave less than 5 per cent of the color produced by an equivalent weight of DHA, and are therefore considered to be negative.

It is to be noted from the results presented in Table IV that, with the exception of DHA and its simple derivatives, only four *ketonic* steroids gave a positive color test. Two of these, namely Δ^5-3(β)-chloroandrostenone-17 and $\Delta^{3,5}$-androstadienone-17, are known to occur in urine extracts, and are generally believed to be transformation products derived from DHA in the course of the hydrolytic procedure utilized to free the steroids from their conjugates. The determination of these compounds as DHA would not be erroneous, therefore, and could be advantageous since the formation of artifacts is difficult to control or eliminate. The other two ketonic steroids, pregnenolone and Δ^5-3(β)-hydroxy-21-acetoxy-20-ketopregnene, have not as yet been demonstrated in urine, although pregnenolone has been found in pig testis by Prelog and coworkers (6). 3(β)-21-Dihydroxy-20-ketopregnene has not as yet been isolated from natural sources. Since the remainder of the compounds in Table III are all non-ketonic, they should not interfere with the determination of DHA by the colorimetric procedure, provided that the extract has first been separated into ketonic and non-ketonic fractions by a suitable procedure.

Since the relation between color production and concentration of DHA is not strictly lineal in this procedure, and in the application to urine extracts Δ^5-3(β)-chloroandrostenone-17 and $\Delta^{3,5}$-androstadienone-17 would be determined as DHA, the proportionality between color and concentration of these substances must be established. It can be seen from Fig. 1 that, while DHA and the chloroketone give identical color intensity over the range of concentrations investigated, the slope of the curve relating color and concentration for $\Delta^{3,5}$-androstadienone-17 departs markedly from that of DHA. While at 10 γ the agreement with DHA is excellent, with increasing concentration of dienone a relatively lower color intensity is developed, a result very probably to be ascribed to the greater destruction of the doubly unsaturated compound under the drastic conditions of the reaction. In order to test whether low concentrations of $\Delta^{3,5}$-androstadienone-17 could be accurately assayed in the presence of DHA and 3(β)-chloroandrostenone-17, a mixture of these three substances in equal proportions was prepared and assayed at 10 and 30 γ levels. In both instances the calculated value was obtained for the mixture, and it is therefore apparent that unless $\Delta^{3,5}$-androstadienone constitutes more than one-third of the chromogenic steroids in the color reaction no serious deviation from the true value is encountered. For greatest accuracy with higher concentrations of the dienone the assay must be conducted with amounts of about 10 γ. A distinction between DHA and the chloroketone or the dienone can be obtained by separation of the ketonic fraction into α and β subfractions by precipitation with digitonin. Of the three compounds only DHA is precipitable with digitonin, and therefore the two transformation products will be found in the α-ketonic fraction.

Table VI illustrates the application of the colorimetric determination of DHA to urine extracts. The fractions studied were kindly supplied to us by Dr. Richard Landau of the Department of Medicine, University of Chicago, and Dr. Konrad Dobriner of the Sloan-Kettering Institute, New York. The extracts were selected primarily on the basis of availability and are therefore not to be considered characteristic of either the normal or the diseased state. The values obtained do serve to indicate, however, the fact that chromogenic compounds of the type of DHA and its transformation products constitute an appreciable percentage of the ketosteroid fraction of urine. It is noteworthy that in each instance in which α- and β-ketonic fractions from the same urine have been assayed, a considerably higher value is obtained in the α-ketonic fraction than in the β-ketonic fraction. The exception is a patient with an adrenal tumor in which the excretion of DHA was at a very high level. Even under these circumstances approximately 40 per cent of the total chromogen was obtained in the α-ketonic fraction. In view of the specificity of the color reaction it may therefore be suggested that the procedures used in the preparation of these urine extracts have resulted in the conversion of a considerable portion of DHA to the chloroketone and the dienone.

TABLE VI

Colorimetric Estimation of Dehydroisoandrosterone in Ketonic Fraction of Urine Extracts

Ketosteroids*			Dehydroisoandrosterone equivalents		
Total ketonic fraction	α-Ketonic fraction	β-Ketonic fraction	Total ketonic fraction	α-Ketonic fraction	β-Ketonic fraction
mg.	mg.	mg.	mg.	mg.	mg.
8.5			2.2		
7.6			1.1		
2.8			0.3		
0.44			0.07		
0.23			0.05		
0.21			0.04		
	5.9	0.1		0.24	0.03
	7.0	1.2		0.93	0.27
	8.8	0.28		0.56	0
	5.1	0.16		0.35	0.05
	35.0†	30.0		10.1	26.2

* Measured by the Holtorff and Koch (7) modification of the Zimmermann (8) reaction.

† Patient with an adrenal tumor.

DISCUSSION

From the results obtained with neutral steroids tested and reported here, as well as with the acidic and neutral compounds investigated by Kerr and Hoehn (2), the structural requirements for a positive Pettenkofer test can be generalized. Unsaturation in Ring B, or a group which can give rise to unsaturation in Ring B, and a hydroxyl group or double bond in Ring A are both essential for the reaction. Of the compounds tested, the simplest substance conforming with these requirements is Δ^5-3(β)-hydroxyandrostene, which is approximately one-half as chromogenic as DHA. All other substances giving a positive reaction possess an additional functional group in the molecule. Thus cholesterol, which differs from 3(β)-hydroxy-Δ^5-androstene only by the aliphatic side chain at C-17, is non-chromogenic, while 3(β)-hydroxy-Δ^5-lithocholenic acid gave a positive test (2). The intensity of color is thus markedly influenced by the other functional groups in the molecule and an approximation of this effect may be made. A carboxyl group at C-24 is equivalent to a ketone group at C-17; a ketol at C-20,C-21 increases the chromogenic effect; a ketone at C-20 is less effective than a C-17 ketone, and replacement of a ketone by a hydroxyl group at the same position decreases the color intensity; two hydroxyl groups in Ring D are approximately equivalent to a C-20 ketone in the color reaction. It should be noted that Δ^{16}-3(α),20(β)-dihydroxyallopregnene gave a positive test, whereas $\Delta^{5,16}$-3,4-dihydroxy-20-ketopregnadiene was negative. The color reaction, therefore, while not completely specific, is qualitatively indicative of a C-3 hydroxyl together with actual or potential unsaturation in Ring B and oxidation elsewhere in the molecule.

The application of this color test to the non-ketonic fraction of urinary extracts should prove of value, since isolation experiments have shown that chromogenic compounds such as Δ^5-3,17-dihydroxyandrostene and Δ^5-3,16,17-trihydroxyandrostene are present in this fraction. Further investigation of the specificity of the reaction would be desirable before any considerable reliance could be placed on the method as a means of identification of individual non-ketonic steroids, but extension to this relatively unexplored fraction should not be overlooked.

We wish to express our appreciation for the advice and encouragement received from the late Professor F. C. Koch throughout the course of this investigation. We also acknowledge the technical assistance of Mr. Arthur A. Wykes, and thank the Wm. S. Merrell Company, Cincinnati, and Dr. Robert S. Shelton, Vice President, for permission to use their laboratory facilities during the summer of 1943. Among the investigators to whom we are indebted for samples of crystalline steroids are Dr. Wayne Cole, Dr. Lewis Engel, Dr. Seymour Lieberman, Dr. H. L. Mason, Dr. A. D. Odell, Dr. R. E. Marker, and Dr. C. R. Scholz.

SUMMARY

1. A colorimetric analytical method for dehydroisoandrosterone (DHA),

which is applicable to urine extracts as well as to pure solutions, has been described. It is based on the color produced when acetic acid solutions containing 10 to 50 γ of the steroid are treated at 67° with furfural and sulfuric acid.

2. The specificity of the method has been investigated with 56 different crystalline neutral steroids. Forty of the compounds gave no significant color.

3. The sixteen compounds which gave positive tests may be divided as follows: (*a*) DHA and simple derivatives including the transformation products Δ^5-3(β)-chloroandrostenone-17 and $\Delta^{3,5}$-androstadienone-17, derived from DHA during acid hydrolysis of urine; (*b*) two other neutral ketones, pregnenolone and 21-acetoxy pregnenolone, which have not as yet been shown to occur in urine; and (*c*) non-ketonic unsaturated steroids.

4. In general the color test indicates the presence of unsaturation or potential unsaturation in Ring B and a hydroxyl group or double bond in Ring A.

BIBLIOGRAPHY

1. Gallagher, T. F., in Moulton, F. R., The chemistry and physiology of hormones, Washington, 186 (1944).
2. Kerr, G. W., and Hoehn, W. M., *Arch. Biochem.*, **4**, 155 (1944).
3. Reinhold, J. G., and Wilson, D. W., *J. Biol. Chem.*, **96**, 637 (1932).
4. Schmidt, L. H., and Hughes, H. B., *J. Biol. Chem.*, **143**, 771 (1942).
5. Irvin, J. L., Johnston, C. G., and Kopala, J., *J. Biol. Chem.*, **153**, 439 (1944).
6. Prelog, V., Tagmann, E., Lieberman, S., and Ruzicka, L., *Helv. chim. acta*, **30**, 1080 (1947).
7. Holtorff, A. F., and Koch, F. C., *J. Biol. Chem.*, **135**, 377 (1940).
8. Zimmermann, W., *Z. physiol. Chem.*, **233**, 257 (1935).

THE EFFECT OF pH UPON THE TOXICITY OF IODOACETIC ACID TO YEAST CELLS*

By J. G. ALDOUS

(From the Department of Pharmacology, Dalhousie University, Halifax, Nova Scotia)

(Received for publication, May 28, 1948)

In investigating the respiratory metabolism of cells whose growth has been inhibited by the use of various agents, it is often advantageous for the action of the inhibitor to be irreversible. Following an exposure to an inhibitory agent of this type, the cells may be freed from the medium containing the inhibitor, thus permitting an investigation of the altered metabolism under a variety of environmental conditions.

The fact that monoiodoacetic acid is a potent inhibitor of fermentation was demonstrated many years ago by Lundsgaard (1). That this inhibition was dependent upon pH was noted by several workers. Ehrenfest (2) reported that yeast fermentation was totally inhibited by 3.6×10^{-4} M at pH 4.6 but that this inhibition gradually decreased as the medium was made more alkaline, until at a pH of 7.0 no inhibition was apparent. She concluded that changes in cell permeability were responsible for these results and produced evidence purporting to support this conclusion.

Following these early observations iodoacetic acid was widely used as a tool for investigating the mechanism of carbohydrate metabolism in a variety of cells and tissue preparations. In spite of this work there resulted little unanimity of opinion as to the specific mode of action of the compound. Both Dickens (3) and Lohmann (4) showed that iodoacetic acid inhibited glyoxalase in liver slices and, from the fact that reduced glutathione restored the activity of their inhibited preparations, concluded that —SH groups were involved in the inhibition. This work was carried on at a pH of 7.4.

With the exception of a paper by Cayrol (5) little appears to have been reported regarding the toxicity of iodoacetic acid. This author observed that in acid media respiration, fermentation, and cell division were inhibited, while at neutrality only fermentation was effected by the same concentration of inhibitor.

Recently Kinsey and Grant (6) classified iodoacetic acid as a "reversible" inhibitor because the depression of growth and respiration observed while the drug was in contact with the cells disappeared upon resuspending the cells in fresh medium. The pH of the medium here was 5.5.

Work with iodoacetic acid has been in progress in this laboratory for a

---

* This investigation was supported by funds from the National Cancer Institute of Canada.

year, and under the experimental conditions employed the effect of the inhibitor was always irreversible. Since exposures of the yeast suspensions to the poison were always made at a pH of 4.5 and under these conditions the results were at a variance with those reported at slightly more alkaline conditions (pH 5.5), it was decided to investigate in detail the question of whether acidity of the medium was responsible for these inconsistencies. These studies emphasize the fact that pH has a marked effect both upon the dissociation of iodoacetic acid and upon the toxicity of the inhibitor to yeast cells, especially over the range, pH 3.5 to 5.5. Moreover, the toxicity appears to be directly proportional to the concentration of the undissociated iodoacetic acid molecules.

Materials and Methods

Method A—Cells of *Saccharomyces cerevisiae* originally obtained from a single cell isolation were grown for 18 hours on plates of Miller (7) agar medium incubated at 20°. After harvesting, the cells were washed twice by centrifugation with phosphate buffer (0.05 M KH_2PO_4) and were finally made up as a 1 per cent suspension in phosphate buffer. Aliquots of this suspension were then centrifuged and the packed cells resuspended in phosphate buffer adjusted to pH values of 3.5, 4.0, 4.5, 5.0, and 5.5. 5 ml. of each suspension were placed in each of five L-shaped test-tubes immersed in a constant temperature bath at 20°. 3 ml. of phosphate buffer at the corresponding pH were added plus 1 ml. of 10 per cent solution of glucose in phosphate buffer adjusted to the corresponding pH. This mixture was shaken for ½ hour, at which time 1 ml. of 10^{-2} M iodoacetic acid (pH 4.5) was added to each suspension. A sixth aliquot to which no iodoacetic acid was added served as a control. After ½ hour each tube was sampled for viable cells, the procedure being that of serial dilution. Sampling, diluting, and the final plating out into the Miller gelatin medium were all carried out in duplicate.

In two experiments in this series, the KH_2PO_4 buffer was replaced by the McIlvaine citric acid-phosphate buffer after it had been established that this procedure did not alter the characteristics of the respiratory metabolism. Identical results were obtained in both cases.

Results

Viable cell counts carried out according to the above procedure varied from 0 to 100 per cent (per cent of the control colony count) as the pH was increased from 3.5 to 5.5. The actual figures are recorded in Table I, from which it is immediately apparent that the greatest toxicity is produced in the most acid environment.

The possibility that these results might be due to varying degrees of dissociation of the iodoacetic acid was then considered. Accordingly, the percentage dissociation of the acid at various points throughout the range covered in the above experiments was calculated, and, since toxicity decreases with increasing dissociation, the per cent *undissociated acid* was plotted against pH. Such a plot is indicated in Fig. 1.

When viability is plotted against concentration of undissociated acid, as has been done in Fig. 2, one obtains a fair approximation to a straight line. This may be taken as an indication that the irreversible toxic action of iodoacetic acid is directly proportional to the concentration of the undissociated molecule. Confirmatory evidence for this conclusion was sought by an entirely different procedure based upon the following reasoning.

TABLE I

Influence of pH upon Toxicity of Iodoacetic Acid

The amount of iodoacetic acid added in all cases was 1 ml. of 10^{-2} M to 9 ml. of yeast suspension. The per cent viability represents the per cent of the control (untreated) colony count. Undissociated acid concentrations were computed from Fig. 1.

pH*	Per cent viability	Undissociated acid, $\times 10^{-4}$ M
3.5	0	8.0
3.7	0	7.0
3.9	0	6.0
4.1	5	5.0
4.35	15	3.8
4.5	40	3.1
4.85	81	1.6
5.0	100	1.2
5.45	90	0.4
5.5	100	0.4

* pH measurements taken immediately prior to addition of iodoacetic acid to yeast suspension.

If the concentration of the undissociated acid is the factor responsible for the toxicity of the drug, then one should be able to expose the cells to varying concentrations of iodoacetic acid under conditions of constant pH and observe much the same phenomenon. Moreover, since the degree of dissociation at any pH is known, the viable cell counts from such an experiment, when plotted against the concentration of undissociated acid (rather than against concentration of the acid added), should fall along the same line as that shown in Fig. 2. A series of such experiments was carried out as indicated below.

Method B—The harvested, washed cells were made up as a 1 per cent suspension in phosphate buffer, pH 4.5. Each L tube received 5 ml. of

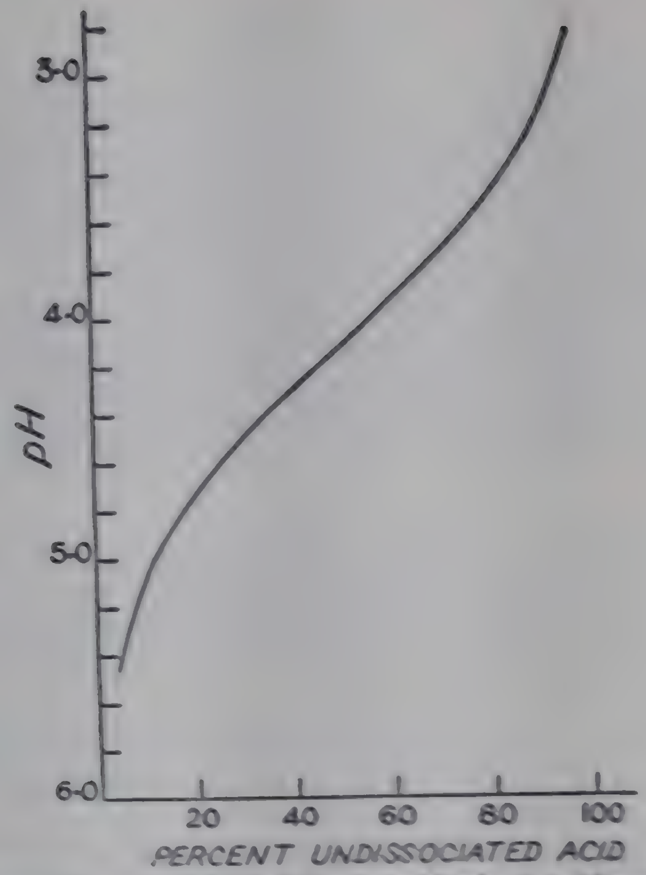


FIG. 1. The dissociation curve for iodoacetic acid calculated from $K_a = 7.5 \times 10^{-5}$

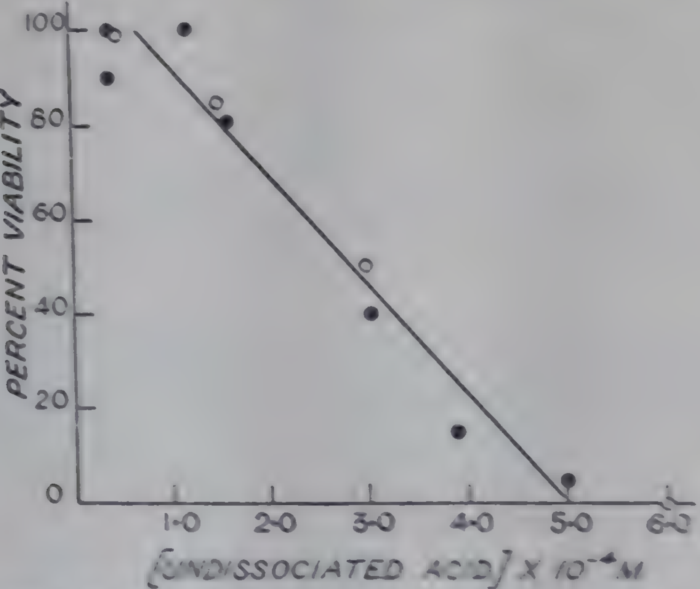


FIG. 2. Viability of yeast cells as a function of the concentration of undissociated iodoacetic acid. ●, the result from experiments in which the undissociated acid concentration was varied by changing the pH of the medium (Method A). ○, the resul from experiments carried out under conditions of constant pH, the undissociated acid concentration being varied by changing the concentration of the inhibitor that wa added to the yeast suspension (Method B).

yeast suspension, various amounts of phosphate buffer, and 1 ml. of 10 per cent glucose solution. After the ½ hour equilibration period, various quantities of 10^{-2} M iodoacetic acid (to make the total volume of each tube to 10 ml.) were added and the whole mixture shaken for ½ hour prior to sampling for viable cells. In some experiments in this series the KH_2PO_4 was replaced by the citric acid-phosphate buffer (pH 4.5) with no change in results. The final concentration of iodoacetic acid in this series ranged from 2×10^{-4} M to 2×10^{-3} M. Since, at a pH of 4.5, 30 per cent of the acid exists in the undissociated form, each of the concentrations of acid used was multiplied by 0.3 and the resulting values from the four experiments together with corresponding viabilities were plotted in Fig. 2.

DISCUSSION

It will be noted that the points in Fig. 2 show a certain amount of scatter about the line intended to represent their average. This situation is largely due to the difficulty in maintaining the pH at any given value in this acid range.

The maintenance of constant pH in a suspension of yeast cells that are actively metabolizing glucose presents a difficult problem, especially if one seeks to use a buffer which does not modify the metabolic characteristics of the cells. Potassium dihydrogen phosphate is a poor buffer over the range covered in the above experiments, and its use necessitated frequent pH determinations. For this reason all of the experiments were repeated with the McIlvaine citric acid-sodium phosphate buffer, which was found to maintain the pH of the medium at its original value over reasonably long periods of time.

The interpretation of the data presented in Fig. 2 is fairly obvious, although it is somewhat surprising to find that the undissociated molecule rather than the iodoacetyl ion is the species responsible for the irreversible toxicity.

Preliminary measurements of the respiratory characteristics of the suspensions treated even with low concentrations of iodoacetic acid show almost a complete absence of both aerobic respiration (oxygen consumption and CO_2 production) and anaerobic fermentation. This association of irreversible cellular damage with cessation of respiratory activity is interesting when compared to the situation obtaining under more alkaline conditions. In those experiments in which an exposure to iodoacetic acid was made at a pH of 5.5, all the cells developed normally and showed very little change in respiratory activity. Nevertheless, under these and more alkaline conditions, when the iodoacetyl ion predominates, a well defined inhibition of respiration has been noted as long as the toxic ion is present in the medium. Here, however, there appears to be a selectivity of action associated with the ion, since CO_2 production is depressed but oxygen consumption frequently remains unaltered. This would seem to point to a difference in specificity of action between the ion and the molecule, although one must also consider the possibility that irreversible cellular damage (obtained under acid conditions) may in itself lead to cessation of all respiratory activity. Certainly the above experiments indicate that the iodoacetic acid molecule has a much greater affinity for the cell than does the iodoacetyl ion.

SUMMARY

As a result of experiments performed under conditions of varying pH and of varying iodoacetic acid concentration it has been demonstrated that the irreversible toxic action of the inhibitor toward yeast cells is proportional to the concentration of undissociated iodoacetic acid. At pH values lower than 5.0 the action of the inhibitor is irreversible, while in media more alkaline than 5.0 the action is reversible.

BIBLIOGRAPHY

1. Lundsgaard, E., *Biochem. Z.*, **220,** 1 (1933).
2. Ehrenfest, E., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **97,** p. lxxvi (1932).
3. Dickens, F., *Biochem. J.*, **27,** 1141 (1933).
4. Lohmann, K., *Biochem. Z.*, **262,** 152 (1933).
5. Cayrol, P., *Ann. Physiol. et Physicochemie Biol.*, **9,** 999 (1933).
6. Kinsey, V. E., and Grant, W. M., *J. Cell. and Comp. Physiol.*, **30,** 31 (1947).
7. Miller, W. L., Eastcott, E. V., and Maconachie, J. E., *J. Am. Chem. Soc.*, **55,** 1502 (1933).

STUDIES ON AMINO ACID EXCRETION IN MAN

II. AMINO ACIDS IN FECES*

By A. LEONARD SHEFFNER, JOSEPH B. KIRSNER, and
WALTER L. PALMER

(From the Frank Billings Medical Clinic, Department of Medicine, University of Chicago, Chicago)

(Received for publication, June 17, 1948)

The fecal nitrogen of normal man remains relatively unchanged despite considerable variation in protein nitrogen intake, provided that the dried weight of food is kept constant (1). Whether or not the fecal amino acids maintain a similar constancy despite variation in the amino acid content of the diet has not been adequately investigated heretofore.

The purpose of this paper is to report the data obtained by microbiological measurement of eight amino acids in the food and in the feces of two healthy, adult men fed a variety of diets. The preparation of feces samples for amino acid analysis by the microbiological technique also is described.

Analyses were performed on acid-hydrolyzed samples, and therefore the total amounts of the amino acids methionine, lysine, arginine, histidine, leucine, isoleucine, valine, and threonine were measured. The results indicate that, despite great differences in the amounts ingested, the quantity of individual amino acids as well as the total nitrogen in the feces remains relatively constant.

EXPERIMENTAL

As described previously (2), the subjects were two healthy young men, hospitalized for the purposes of this investigation in the metabolism section of the Albert Merritt Billings Hospital. They were permitted normal activity. Each diet was studied for two consecutive 6 day periods, following a 3 day interval which permitted adjustment of the subjects to the change in food. Though the intake of protein was varied, the caloric intake and the weight of dried matter were kept as uniform as possible throughout the study by adjustment of the carbohydrate content of the diet. Sufficient amounts of carbohydrate, fat, and vitamin supplements were administered to make the diets nutritionally complete. The preparation and

---

* A portion of this study was presented before the Division of Biological Chemistry at the Chicago meeting of the American Chemical Society, April, 1948.

This study was supported in part by grants from the Evaporated Milk Association and the American Dairy Association.

analysis of the food and the microbiological procedure utilized in this study have been described in a previous publication (2).

Except in Periods 9 and 10, in which crystalline methionine was added to the diet, the amount of individual amino acids ingested was varied by changing either the quantity or type of protein in the food. With the exception of methionine, no attempt was made to study the effects of added individual amino acids upon the excretion in feces of the other amino acids.

Collection and Preparation of Feces Samples for Analysis—Feces were collected into a large, weighed, covered glass mixing bowl and stored in the refrigerator for each 6 day period. There was no obvious decomposition of the feces through growth of bacteria or mold. Each 6 day collection was marked by 0.3 gm. of carmine administered to the subjects just prior to the first meal of the period and at the end of exactly 6 days; the feces between the two markers were saved for analysis.

The total collection of feces for each period was weighed and homogenized with distilled water in a Waring blendor. Measured aliquots were then taken for total nitrogen and for amino acid analysis. Total nitrogen was determined by the semimicro-Kjeldahl procedure with selenium dioxide and cupric sulfate as the digestion catalysts.

For amino acid analysis, 2.5 to 3 gm. of the homogenized sample were weighed accurately into a conical beaker and autoclaved with 20 ml. of 4 N hydrochloric acid for 10 hours at 120°. The samples were then boiled 10 minutes with 250 mg. of norit A, filtered by suction, adjusted to pH 6.8, and made up to a volume of 1 liter. All samples were hydrolyzed in duplicate and each hydrolysate was analyzed at three different levels of concentration.

The norit A treatment is important in the preparation of the samples because it removes certain unidentified substances in feces which stimulate the growth of the assay organisms and cause apparently excessive recoveries of added amino acids. Recovery of pure amino acids added to normal human feces before hydrolysis ranged from 93 to 108 per cent.

RESULTS AND DISCUSSION

The data for the eight amino acids are presented in Table I. Since there were no significant differences between the results for each subject, the values for both in each period were averaged together. The fecal nitrogen remained relatively constant throughout the study.

The intake of histidine per 6 day period ranged from 6.9 to 13.8 gm. The variation in the intake of the remaining seven amino acids was greater, being highest for lysine, 13.2 to 31.7 gm. per period. Nevertheless, the total output of each amino acid in the feces per 6 day period did not vary more than 0.55 gm., plus or minus, from the mean output for the particular amino acid. The values for methionine in the feces never varied more than 0.2 gm. from the mean, even when 6.0 gm. of DL-methionine were added to a diet adequate to maintain the subjects in positive nitrogen balance.

TABLE I

Microbiologically Available Amino Acids in Feces of Two Adult Men Fed Various Diets

Each value represents the averaged result for the two subjects studied for a 6 day period.

Period No.	Methionine		Lysine		Arginine		Histidine	
	Ingested	In feces	Ingested	In feces	Ingested	In feces	Ingested	In feces
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	11.5	0.8	31.7	2.9	26.0	1.9	13.8	0.7
2	11.5	0.6	31.7	2.6	26.0	2.1	13.8	0.8
3	6.4	0.7	13.2	2.1	13.3	1.3	6.9	0.6
4	6.4	0.8	13.2	2.6	13.3	1.6	6.9	0.7
5	11.0	0.7	30.8	2.3	24.7	1.6	12.7	0.7
6	11.0	0.7	30.8	2.4	24.7	1.4	12.7	0.7
7	8.8	0.5	23.8	1.9	23.6	1.2	11.9	0.7
8	8.8	0.7	23.8	2.2	23.6	1.5	11.9	0.7
9*	14.8	0.9	23.8	2.5	23.6	1.7	11.9	0.9
10*	14.8	0.7	23.8	2.3	23.6	1.6	11.9	0.7

Period No.	Leucine		Isoleucine		Valine		Threonine	
	Ingested	In feces	Ingested	In feces	Ingested	In feces	Ingested	In feces
1	39.0	2.9	28.4	2.3	27.5	2.6	19.8	2.2
2	39.0	2.5	28.4	2.0	27.5	2.4	19.8	1.9
3	24.7	2.2	17.1	1.6	16.9	1.9	11.5	1.5
4	24.7	2.5	17.1	1.8	16.9	2.0	11.5	1.4
5	37.0	1.8	27.3	1.5	24.5	1.5	18.5	1.6
6	37.0	1.8	27.3	1.7	24.5	1.8	18.5	1.7
7	34.7	2.0	22.2	1.6	23.0	1.5	17.2	1.4
8	34.7	2.4	22.2	1.4	23.0	1.6	17.2	1.6
9*	34.7	2.8	22.2	2.2	23.0	2.2	17.2	1.7
10*	34.7	2.4	22.2	1.7	23.0	1.9	17.2	1.7

* 6 gm. of DL-methionine added per period, supplementing the diet fed during Periods 7 and 8.

To study the other extreme, one of the subjects was later placed on a diet, the major protein of which was supplied by a peptone solution treated with 3 per cent hydrogen peroxide to destroy some of its methionine. This diet was followed by a regimen in which the peptone was treated with 30 per cent hydrogen peroxide. In both instances the daily diet was supplemented by 1.25 gm. each of DL-tryptophan, L-tyrosine, and L-cystine to compensate for the destruction of these amino acids by the peroxide. The total quantity of methionine ingested was only 2.3 gm. in the first 6 day period and 0.9 gm. in the latter. The amount of methionine excreted in the feces in each period was 0.9 and 0.6 gm. respectively, values within 0.2 of the mean established on diets containing adequate quantities of methionine. Although the hydrogen peroxide-treated peptone is relatively low also in histidine and leucine, the quantities of these amino acids in the feces likewise did not decrease during the ingestion of the peptone diet.

It is apparent, therefore, that the quantity of amino acids excreted in the feces remains comparatively constant despite relatively large changes in the individual amino acid content of the diet. Also, analysis of the dilute acid extract of feces, deproteinized by tungstic acid precipitation, reveals minute, if any, amino acid activity microbiologically, and hydrolysis of the protein-free extract does not increase this activity. Therefore, practically none of the fecal amino acids can be considered as free, polypeptide, or conjugated amino acids.

No conclusions can be made concerning the direct source of fecal amino acids on the basis of the data obtained in this study. However, the results indicate that the nitrogen represented by any of the eight amino acids studied maintains a fairly constant proportion to total fecal nitrogen. This finding is in agreement with the commonly accepted thesis that the greatest portion of fecal nitrogen is in the form of bacterial protein and that the intestinal bacteria produce protein of constant composition. In addition, if the intestinal bacteria derive nutrition from unabsorbed proteins and amino acids, variation in the amino acid content of the diet, although causing no change in the composition of bacterial protein, would, however, produce a variation in the total quantity of protein in the feces. The relatively constant excretion in the feces of the eight amino acids studied indicates, therefore, that the fecal amino acids are not unabsorbed residues of the diet. Presumably, they represent part of a constant supply of protein, probably digestive enzymes, secreted into the lumen of the gastrointestinal tract and excreted as such or converted into bacterial protein before elimination from the body.

SUMMARY

1. The total quantity of each of the amino acids methionine, lysine, arginine, histidine, leucine, isoleucine, valine, and threonine was measured microbiologically in the food and feces of two normal adult men.

2. The special preparation of feces samples for the amino acid analysis by the microbiological technique is described.

3. The results indicate that, despite great differences in the amounts fed, the total quantity of individual amino acids excreted in the feces remains comparatively constant.

4. It is suggested that these fecal amino acids represent amino acids secreted into the lumen of the gastrointestinal tract as components of digestive enzymes, excreted as such or converted into bacterial protein before elimination from the body.

The authors wish to express their gratitude to Miss Blanche Parish, R. N., for supervising the collections and care of the subjects, to Miss Minnie Brandt for composing and preparing the diets, and to the subjects, Mr. John Doull and Mr. Richard Herz, for their cooperation.

BIBLIOGRAPHY

1. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry; Interpretations, Baltimore, 2nd edition (1946).
2. Sheffner, A. L., Kirsner, J. B., and Palmer, W. L., *J. Biol. Chem.*, **175**, 107 (1948).

STUDIES ON THE CYCLOPHORASE SYSTEM

II. THE COMPLETE OXIDATION OF FATTY ACIDS

By ALLAN L. GRAFFLIN* and D. E. GREEN†

(From the Enzyme Laboratory, Department of Medicine, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, April 28, 1948)

In the first paper of this series (1) a detailed description was given of the method of preparation and the properties of the cyclophorase system from rabbit kidney and liver which catalyzed the complete oxidation of pyruvic acid to carbon dioxide and water by way of the citric acid cycle. The present communication deals with the complete oxidation of fatty acids to carbon dioxide and water as catalyzed by the same cyclophorase-containing preparations from kidney and liver.

Leloir and Muñoz (2–4) were the first to achieve β oxidation of fatty acids in cell-free preparations from animal tissues. If we consider the extreme instability of the system from rat and guinea pig liver with which they worked, it is indeed remarkable that they should have discovered essentially all the important requirements for activity of the enzyme system. They established the necessity for the following four components: (*a*) magnesium ions, (*b*) phosphate ions, (*c*) adenylic acid or adenosine triphosphate, and (*d*) cytochrome *c*. They recognized that the oxidation of fatty acids had to be primed or sparked, as it were, by the simultaneous oxidation of one of the intermediates in the citric acid cycle. They observed that coincident with the oxidation of fatty acids inorganic phosphate was taken up while a substance answering the description of phosphopyruvic acid accumulated. Under one set of conditions, butyric acid was oxidized only as far as acetoacetic acid, while under other conditions, or even without a change in the conditions, butyric acid was oxidized beyond the stage of acetoacetic acid. Leloir and Muñoz found that fatty acids from C_4 to C_8 were oxidized most rapidly in their liver mince.

More recently the problem has been taken up by Lehninger (5–10), who has described a malonate-insensitive system in liver which catalyzes the oxidation of octanoate and other fatty acids quantitatively to acetoacetic acid. The malonate insensitivity of Lehninger's system contrasts with the malonate sensitivity of the Leloir and Muñoz system. Lehninger has also observed that the oxidation of fatty acids can be sparked by the cooxidation of α-ketoglutarate, but he has claimed in addition that adenosine triphosphate in large excess can replace the cooxidation system. The necessity for adenosine triphosphate led Lehninger to the view that the fatty acids were phosphorylated enzymatically with the formation of acyl phosphates.

---

* Senior Fellow in Cancer Research, American Cancer Society, upon recommendation of the Committee on Growth of the National Research Council. Present address, Department of Anatomy, School of Medicine, The Johns Hopkins University, Baltimore, Maryland.

† Present address, Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin.

Results

Oxidation of Fatty Acids by Kidney

Components of Fatty Acid Oxidation System—The kidney enzyme system prepared as described under "Experimental" will oxidize fatty acids when fortified with adenosine triphosphate (or adenosine monophosphate), magnesium ions, and inorganic phosphate. In numerous studies with acetic, butyric, β-hydroxybutyric, crotonic, vinylacetic, and β-ketocaproic acids, it was observed that the rate of fatty acid oxidation may be strikingly reduced in the absence of any one of these additions. The magnitude of these effects is highly variable. With a very fresh enzyme preparation they may be absent or slight, or appear only rather late in the experiment. However, with a preparation even a few hours old they are usually apparent at the very beginning of the experiment, and become more marked as the experiment proceeds (Fig. 1).

Rather early in the present investigation it was noted that the rates of oxidation of various fatty acids, in the presence of adenosine triphosphate, magnesium, and phosphate, may frequently be increased significantly by adding to the system a small amount (0.3 cc.) of a 1 per cent aqueous extract of an acetone powder of pig heart (Fig. 1). Under the circumstances, this heart extract was included in the great majority of the experiments here reported. In the following paper of this series, Knox *et al.* (11) have presented evidence that the addition of the extract is equivalent to adding a small amount of the sparker or primer; *i.e.*, a substance which in catalytic amount potentiates the oxidation of a fatty acid.

Oxidation of Fatty Acids by Kidney Preparations—Under the experimental conditions obtaining for Table I, kidney enzyme preparations have been found to oxidize rapidly (*a*) all of the saturated straight chain monocarboxylic fatty acids from acetic through *n*-tridecylic acid with the exception of propionic acid, (*b*) some substituted and unsaturated derivatives of these acids, and (*c*) the branched chain fatty acid, isocaproic acid.

Oxygen uptake data from numerous manometric experiments are summarized in Table I, which is largely self-explanatory. Substrates were always added to the main compartment prior to gassing and equilibration, since oxidative activity is markedly reduced or abolished unless the enzyme is protected by substrate during equilibration. Inasmuch as oxidation proceeds at an unknown and presumably variable rate during gassing and equilibration, the total oxygen uptake in such experiments is greater than the measured uptake by an amount which can only be guessed at by extrapolation. As an aid to extrapolation, the rate of oxygen consumption during the first 5 minutes after closing the taps has been recorded in each case.

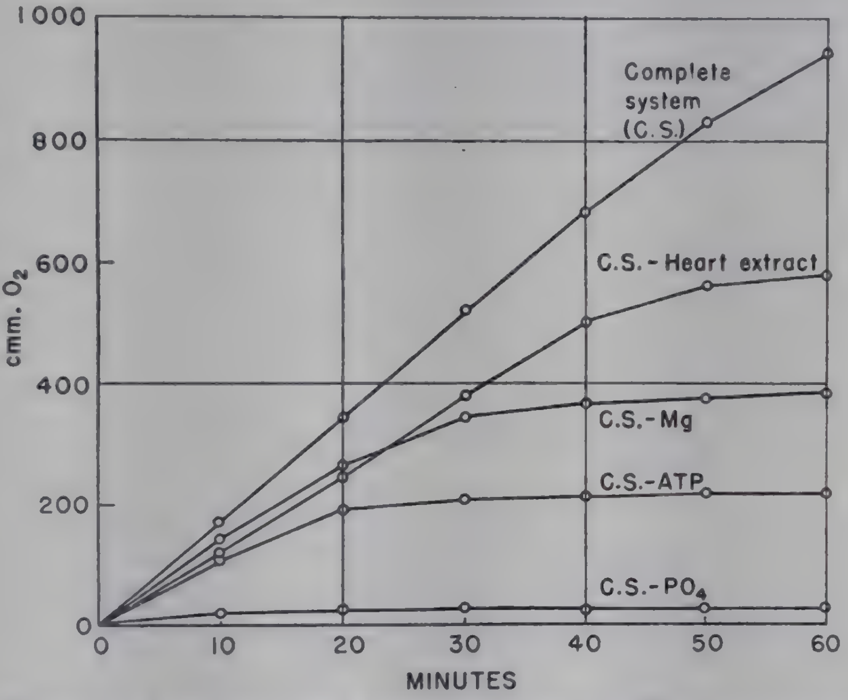


FIG. 1. Oxidation of crotonic acid, component study. The complete system included adenosine triphosphate, magnesium ions, phosphate buffer, heart extract, 30 micromoles of crotonic acid, and 1 cc. of kidney enzyme (R_3K) used 5 hours after preparation. Oxygen uptake in the control without added substrate was 59 c.mm. in 60 minutes.

The data in Table I are consistent with the complete oxidation, to carbon dioxide and water, of all of the straight chain, saturated, unsubstituted fatty acids with an even number of carbon atoms from acetic through *n*-lauric acid, as well as of the following substituted or unsaturated derivatives of these acids: crotonic, vinylacetic, and β-ketocaproic acids. In some instances, *e.g.* acetic (10 micromoles) and vinylacetic acids, the recorded oxygen uptake is significantly in excess of theoretical requirements for complete oxidation, and the disparity becomes even more marked with extrapolation for the equilibration period. These high values are interpreted as indicating the inadequacy of the correction for oxygen uptake in the control without added substrate. This correction is based upon the

Table I

On Completeness of Fatty Acid Oxidation by Kidney System

In all the manometric experiments, the following additions were made: 0.3 cc. of 0.01 M adenosine triphosphate, 0.2 cc. of 0.02 M magnesium sulfate, 0.5 cc. of 0.04 M phosphate buffer of pH 7.3, 0.3 cc. of heart extract, substrate, and 2 cc. of enzyme (R_3K added last) used immediately after preparation; alkali in the center well and 100 per cent oxygen in the gas phase; acids added in the form of neutralized sodium salts. The interval between the entry of the manometer into the bath at 38° and the closing of the stop-cock was timed and kept to 5 minutes. The values for recorded oxygen uptake have all been corrected for the oxygen uptake of the blank experiment without added substrate. All experiments were carried to completion, *i.e.* until oxygen uptake was at a standstill. The experiments lasted from 70 to 105 minutes in all but one instance (140 minutes). The total oxygen uptake in the controls without added substrate varied from 7 to 20 microatoms in the various experiments.

	Fatty acid	Oxygen uptake, *microatoms*			Theory, *microatoms oxygen*	
		Micromoles added	Recorded in 1st 5 min.	Total recorded	For complete oxidation to CO_2 and H_2O	For oxidation to propionic acid, CO_2 and H_2O
Even numbered	Acetic	10	7.0	47.7	40	
		15	6.0	54.5	60	
	Butyric	5	12.1	47.6	50	
		10	12.6	94.2	100	
		10	12.9	100.1	100	
	n-Caproic	5	11.5	74.6	80	
	Caprylic	5	11.7	99.3	110	
	Capric	1.25	8.8	33.4	35	
		2.50	8.4	66.4	70	
	Lauric	1	7.1	35.6	34	
Odd numbered	*n*-Valeric	5	7.5	30.0	65	30
		5	5.4	28.1	65	30
	Heptylic	2.5	13.8	24.8	47.5	30
		2.5	13.1	24.6	47.5	30
		2.5	12.3	20.7	47.5	30
		5.0	13.9	63.0	95	60
		5.0	16.0	58.5	95	60
		5.0	17.5	53.9	95	60
	Pelargonic	5	9.4	98.0	125	90
	Undecylic	1	10.1	19.6	31	24
		2	11.8	47.1	62	48
	$\Delta^{10,\,11}$-Undecylenic	1	8.4	24.4	30	24*
	Tridecylic	1	5.2	26.5	37	30
Branched chain	Isocaproic	5	5.6	33.0	80	30†
Derivatives	Crotonic	10	7.5	92.3	90	
	Vinylacetic	10	9.1	103.2	90	
	β-Ketocaproic	5	9.6	63.9	70	

* Theory is for oxidation to acrylic acid, CO_2 and H_2O.

† Theory is for oxidation to isobutyric acid, CO_2 and H_2O.

assumption that the endogenous substrates in the system are oxidized at the same rate in the presence as in the absence of added fatty acid. It can be shown that this assumption is not valid in all cases and that a substance like pyruvic acid,[1] which is present in some enzyme preparations, remains unoxidized in the blank but undergoes complete oxidation in the experimental vessel coincident with the oxidation of fatty acids. In such cases, the recorded oxygen uptake would exceed that attributable to oxidation of the fatty acid by an amount which would not be completely corrected for by that recorded in the blank without added substrate.

As mentioned above, propionic acid is completely inactive in the kidney system. The other saturated, straight chain, monocarboxylic fatty acids, from *n*-valeric acid through *n*-tridecylic acid, are all actively oxidized. However, the recorded oxygen uptake in all cases is well below the theoretical requirement for complete oxidation to carbon dioxide and water. The data are consistent with the oxidation of odd numbered fatty acids to propionic acid, carbon dioxide, and water, of isocaproic acid to isobutyric acid, carbon dioxide, and water, and of $\Delta^{10,11}$-undecylenic acid to acrylic acid, carbon dioxide, and water. In Paper IV Atchley (13) has described the use of the counter-current distribution method for demonstrating the formation of non-oxidizable residues resulting from the oxidation of *n*-valeric acid and isocaproic acid.

It will be noted in Table I that the amount (in micromoles) of substrate used in these experiments was never large, and that it was progressively reduced as the length of the carbon chain increased. The reasons for this are twofold. First, the activity of these enzyme preparations is regularly impaired and may be abolished by prolonged shaking at a temperature of 38°. Secondly, some of the fatty acids, particularly the higher members of the series, may be readily oxidized at very low concentrations, but oxidized poorly or not at all at higher concentrations. For example, *n*-capric acid was rapidly oxidized to completion at 1.25 and 2.5 micromoles per cup, but was completely inactive at 5 micromoles per cup; *n*-lauric acid was rapidly oxidized to completion at 1 micromole, was oxidized slowly and incompletely at 2 micromoles, and was entirely inactive at 5 micromoles per cup. Even with *n*-valeric acid, this concentration effect may be readily apparent at 10 micromoles per cup and higher.

A considerable number of fatty acids and their derivatives have been tested in manometric experiments, with negative results. In the straight chain fatty acid series, formic, propionic, myristic, palmitic, stearic, and oleic acids were found not to be oxidized by the kidney system. Isobutyric, isovaleric, acrylic, dimethylacrylic, and $\Delta^{4,5}$-pentenoic acids were also inactive over a wide range of concentrations. No α-substituted fatty acid proved active; *e.g.*, α-hydroxybutyric, α-ketobutyric, and α,β-dihydroxybutyric acids. γ-Hydroxybutyric and 3,4-epoxybutyric acids were studied with particular interest as possible intermediates in the oxidation of butyric acid, in view of the rapid and complete oxidation of vinylacetic acid and succinic semialdehyde.[2] Neither was found to show any activity. The introduction of a methyl or phenyl group in the α or β position renders a fatty acid inactive; *e.g.*, 2-phenylbutyric and 3-methylcrotonic acids. All the dicarboxylic acids tested were inactive; *e.g.*, glutaric, adipic, and azelaic acids.

---

[1] The estimation of pyruvic acid by the salicylaldehyde method of Straub (12) has disclosed that many preparations of the kidney system, even at the R_3 residue stage, form during the experiment as much as 1 micromole of pyruvic acid per cc. of enzyme. The origin of this pyruvic acid has not been ascertained.

Oxidation of Postulated "Intermediates" of β Oxidation—Assuming that Knoop's classical theory of successive β oxidation (14) is applicable to the kidney system under investigation, we would anticipate that β-hydroxy, α,β-unsaturated, and β-keto acids corresponding to any oxidizable fatty acid should also be oxidized. The data of Table II show that in all but one case[3] the postulated "intermediates"[4] of β oxidation are indeed oxidized. What is also of considerable interest is that these "intermediates," whenever tested, have been found to be oxidized to the same end-products as the parent acids. When the oxidation of a fatty acid involves merely the loss of a C_2 unit, and particularly when the product of oxidation is inhibitory, *e.g.* propionic acid from valeric acid, the β or $\Delta^{\alpha,\beta}$ derivatives of the parent acid are oxidized to completion only in the presence of a considerable amount of the sparker, even though the parent acid may get by on minimal amounts. This discrepancy is not unexpected in view of the considerations brought up in foot-note 4. β-Hydroxylauric acid was found to be a profound inhibitor of the fatty acid oxidase even at concentrations less than 0.1 micromole per cc. However, it was possible to demonstrate that it was oxidized to within 75 per cent of completion when highly active preparations of the enzyme were tested. The γ,δ-lactone of triacetic acid, which is the ketonic analogue of sorbic acid, was inactive, whereas sorbic acid was fully active.

---

[2] Succinic semialdehyde is oxidized completely to carbon dioxide and water, as shown by the following experiment: 22.8 micromoles were added at the beginning of the experiment. The observed oxygen uptake due to succinic semialdehyde accounted for the complete oxidation of 13.4 micromoles, and there remained at the end of the experiment 8.8 micromoles (theory, 9.4 micromoles).

[3] According to the manometric data $\Delta^{10,11}$-undecylenic acid is oxidized down to acrylic acid. $\Delta^{4,5}$-Pentenoic acid would be an "intermediate" in this degradation and, therefore, should be oxidized in the kidney system. No success has attended many attempts to show that it undergoes oxidation in the kidney system. The liver system can oxidize acrylic acid to carbon dioxide and water, and it is of interest that $\Delta^{4,5}$-pentenoic acid is readily oxidized in the liver system. Its inactivity in the kidney system may well be due to inhibitory effects either of the acid itself or of its product of oxidation (acrylic acid).

[4] The term "intermediates" is placed in quotation marks since, unlike true intermediates, the substances listed in Table II are not oxidized as such by the enzyme system, but only after they have undergone a transformation brought about by the sparker. In another communication, the nature of this transformation will be considered in some detail.

TABLE II

Completeness of Oxidation of β-Hydroxy, β-Keto, and α,β-Unsaturated Fatty Acids by Kidney System

Details as in Table I, except that heart extract was omitted and 5 micromoles of α-ketoglutaric acid were used as the sparker.

Fatty acid derivative	Micromoles added	Micromoles utilized*	Microatoms oxygen absorbed (blank subtracted)	Theory for† complete oxidation to CO_2 and H_2O	Theory for oxidation to propionic or isobutyric acid, and CO_2 and H_2O
				microatoms O_2	*microatoms O_2*
dl-β-Hydroxybutyric	2.0		20.4	18	
	3.0		26.2	27	
	4.0		33.2	36	
dl-β-Hydroxyvaleric	2.5		11.8		12.5
dl-β-Hydroxyisocaproic	2.0		10.0		10.0
dl-β-Hydroxycaproic	1.5		21.0	22.5	
dl-β-Hydroxyoctanoic	1.0		21.4	21.0	
β-Ketovaleric	2.0		8.1		8.0
β-Ketoisocaproic	10.0	3.30	13.8		13.2
β-Ketooctanoic	7.5	3.84	33.8	38.4	
Isocrotonic	6		62.3	54	
$\Delta^{2,3}$-Pentenoic	5		23.8		25
4-Methyl-$\Delta^{2,3}$-pentenoic	2.5		14		12.5
trans-$\Delta^{2,3}$-Hexenoic	2.0		32.8	30.0	
	4.0		62.3	60.0	
cis-$\Delta^{2,3}$-Hexenoic	3		41.2	45.0	
Sorbic	5		70	70	

* β-Keto acids are not oxidized readily in low concentrations. An excess of β-keto acid was therefore added and the amount which disappeared during the experiment was determined by analysis.

† Theory for *dl*-β-hydroxy fatty acids calculated on the assumption that both the *d* and *l* forms are oxidized.

Both the cis and trans forms of α,β-unsaturated acids are equally active in the two cases in which they have been tested (Table II). In keeping with this lack of specificity towards geometrical isomers is the inability of the oxidase system to distinguish between the *l* and *d* stereochemical forms of β-hydroxy acids. Five *dl* acids have been tested, and in each case both isomers were equally oxidized.

Substituting a triple bond for a double bond leads to an inactive compound. Thus, α,β-hexynoic acid was not oxidized under conditions by which α,β-hexenoic acid was oxidized to completion.

Oxidation of Phenyl Fatty Acids—The classical theory of β oxidation is based upon the results of studies with phenyl fatty acids. It was therefore of considerable interest to determine whether the kidney enzyme system could oxidize phenyl fatty acids in the manner postulated by Knoop (14, 15). The point was not easy to test because of the intense inhibitory action of phenyl fatty acids on the fatty acid oxidase system. Thus, γ-phenylbutyric acid at a concentration of 0.003 M inhibited completely the oxidation of butyric, crotonic, and vinylacetic acids, and with butyric acid was still exerting striking inhibition at a concentration as low as 0.0003 M. In order to test oxidizability, γ-phenylbutyric acid had to be diluted to a point at which reliable manometric measurements were no longer possible. The same difficulty obtained for phenylpropionic acid and cinnamic acid at one end of the phenyl fatty acid series, and for phenylcapric and phenyllauric acids at the other. However, it was possible to demonstrate consistently the oxidation of γ-phenylvaleric and γ-phenyloctanoic acids. The rates of oxidation of these two phenyl fatty acids were not sufficiently rapid to permit quantitative studies, and it remains to be determined how far down the carbon chain the oxidation proceeds.

Sparking or Priming of Fatty Acid Oxidation by Members of Citric Acid Cycle in Kidney Preparations—Fresh enzymes usually oxidize fatty acids with great rapidity. However, when the enzyme is allowed to stand for some hours after preparation, the capacity to oxidize fatty acids is greatly reduced, if not abolished. This capacity can be restored by the addition of primers such as α-ketoglutarate or fumarate (Fig. 2). Similar effects have already been reported by others (2, 4, 7, 10, 16–19).

The sparking of acetoacetic acid has been studied in some detail. At 30 micromoles per cup it is regularly oxidized either very slightly or not at all in the absence of an added sparker, even with the most active enzyme preparations; and just as regularly its oxidation can be sparked by α-ketoglutaric acid. Significant effects are obtained with very small amounts of the sparker, and the oxidative rate is sustained for progressively longer periods as the amount of α-ketoglutaric acid is increased (Fig. 3). In Fig. 3 it will be noted that with 0.5, 1, and 2 micromoles of α-ketoglutaric acid, the reaction having proceeded to virtual completion at 60 minutes, the total oxygen uptake in excess of the appropriate α-ketoglutaric acid blank, and attributable to the oxidation of acetoacetic acid, is in direct proportion to the amount of α-ketoglutaric acid added (0.5:156, 1:301, 2 micromoles:605 c.mm. of O_2). Extrapolation for the period of equilibration does not seriously affect this proportionality.

The following experiment indicates that acetoacetic acid can be completely oxidized to carbon dioxide and water in the presence of α-ketoglutaric acid. 10.5 micromoles of acetoacetic acid were added to each of two manometer cups, only one of which was supplemented with 5 micromoles of α-ketoglutaric acid. In the cup with the sparker the oxygen uptake, corrected for the blank with acetoacetic acid, corresponded to the complete oxidation of 7.1 micromoles. Analysis by the aniline-citrate method (20) showed that 3.6 micromoles of acetoacetic acid remained (theory, 3.4 micromoles). In the cup without an added sparker, the corrected oxygen uptake corresponded to the complete oxidation of only 1.4 micromoles of acetoacetic acid.

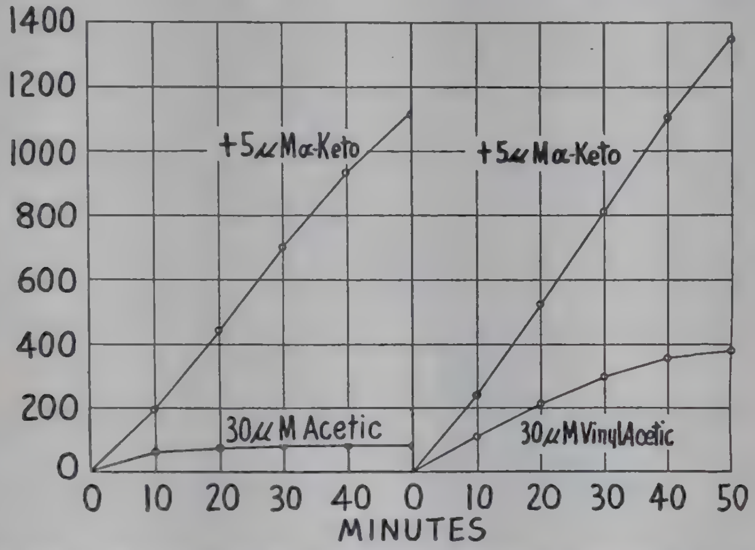


FIG. 2. Sparking of acetic acid and vinylacetic acid oxidation by α-ketoglutaric acid. The complete system was used with 2 cc. of kidney enzyme (R_3K) in the experiment with acetic acid and 1 cc. of enzyme in the experiment with vinylacetic acid. The enzymes were used 5¼ hours and 30 minutes after preparation respectively. The oxygen uptake in appropriate controls (without added substrate and with 5 micromoles of α-ketoglutaric acid) has been subtracted in all cases, and only the excess oxygen uptake attributable to the oxidation of acetic and vinylacetic acids is shown in the figure. The total oxygen uptake in the controls without added substrate was 62 c.mm. in the experiment with acetic acid, and 53 c.mm. in that with vinylacetic acid.

Although most of the sparking experiments were performed with α-ketoglutaric acid, it was found that all members of the tricarboxylic acid cycle, or substances like glutamate or proline which can give rise to members of the citric acid cycle,[5] could effectively spark the oxidation of acetoacetic acid.

The fact that some fatty acids may be readily oxidized at lower concentrations, but oxidized poorly or not at all at higher concentrations, has previously been mentioned. This inhibition at higher concentrations can frequently be overcome by the addition of small amounts of the sparker. A brief description of one experiment will suffice. *n*-Capric acid was rapidly oxidized to completion at 1.25 micromoles and 2.5 micromoles, but was completely inactive at 5 micromoles per cup; on the other hand, 5 micromoles of the acid were oxidized with striking rapidity in the presence of 5 micromoles of α-ketoglutaric acid.

---

[5] Taggart, J. V., and Krakauer, R., unpublished experiments.

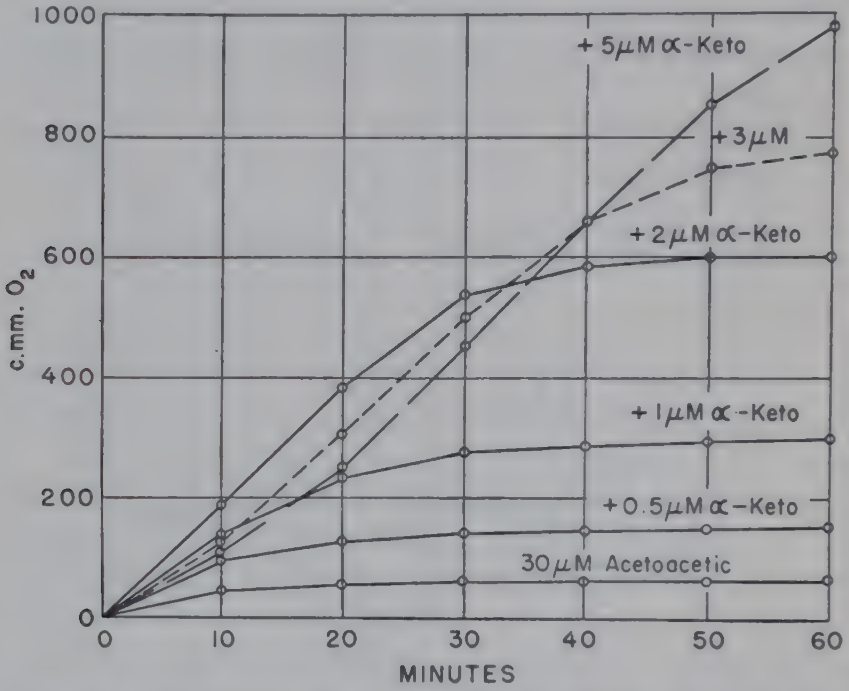


FIG. 3. Sparking of acetoacetic acid oxidation by varying amounts of α-ketoglutaric acid. The complete system was used with 2 cc. of freshly prepared kidney enzyme (R_3K). The oxygen uptake in appropriate controls (without added substrate and with 0.5, 1, 2, 3, and 5 micromoles of α-ketoglutaric acid) has been subtracted in all cases, and only the excess oxygen uptake attributable to the oxidation of acetoacetic acid is shown in the figure. Oxygen uptake in the control without added substrate was 99 c.mm. in 60 minutes.

It may be pointed out that the conditions of Table I are minimal as far as the amount of the sparker is concerned and that the reproducibility of the rates of oxidation was not too good from one enzyme preparation to another. At a higher level of the sparker, reproducibility was excellent. All compounds were therefore also tested in the presence of about 5 micromoles of the sparker. Whenever a fatty acid is referred to as inactive in this communication, it is implied that the test for activity has also been carried out in the presence of an adequate amount of the sparker.

Fatty Acid Oxidation with Ferricyanide—In our previous communication on the cyclophorase system (1), evidence was presented that ferricyanide could replace oxygen as the oxidizing agent and that ferricyanide oxidation could be followed manometrically by measuring carbon dioxide evolution. Ferricyanide has also been found to be effective as an oxidizing agent for fatty acids (Table III). The necessity for the sparking of fatty acid oxidation applies as well to the oxidation by ferricyanide as to the oxidation by molecular oxygen. This particular point is discussed by Knox *et al.* (11).

Inhibition of Fatty Acid Oxidases—At a concentration of 0.003 M, malonate completely inhibits fatty acid oxidation. The same is true for arsenite at the same concentration. 0.004 M barium ions, even in the absence of added inorganic phosphate, abolish the oxidation of butyric acid (10 micromoles per cup). Fluoroacetate, which has been extensively investigated by Bartlett, Barron, and Kalnitsky (21), is an inhibitor of acetic, butyric, and *n*-caproic acid oxidation, and requires further investigation. Propionic acid completely inhibits acetic acid oxidation when the two compounds are present in equimolar concentrations, and markedly inhibits at a concentration one-fourth that of the acetic acid. Butyric acid oxidation is abolished by cinnamic acid in equimolar concentration. Cinnamic acid, at a concentration one-tenth that of the substrate, inhibited the oxidation of butyric and vinylacetic acids about 25 per cent, while that of crotonic acid was inhibited about 75 per cent. γ-Phenylbutyric acid at a concentration of 0.003 M inhibited completely the oxidation of butyric, crotonic, and vinylacetic acids (0.009 M), and striking inhibition of butyric oxidation was observed even at 0.0003 M concentration.

TABLE III

Oxidation of Fatty Acids by Ferricyanide

	Microliters CO_2 per 20 min.		Microliters CO_2 per 20 min.
Control (no fatty acid)	135	*dl*-β-Hydroxybutyrate	510
Acetate	513	Acetoacetate	535
Valerate	253	*cis*-Hexenoate	332
β-Ketovalerate	433	β-Ketohexanoate	831
Butyrate	634	Sorbate	700
Crotonate	484	Caproate	600
Vinylacetate	524		

Each manometer cup contained 1.5 cc. of kidney enzyme at the 3rd residue stage, 0.3 cc. of 0.01 M adenosine triphosphate, 0.2 cc. of 0.02 M magnesium sulfate, 0.1 cc. of 0.5 M sodium bicarbonate, 0.2 cc. of 0.5 M sodium ferricyanide, 0.2 cc. of 0.01 M α-ketoglutarate, and, except for the control, 0.3 cc. of 0.1 M fatty acid. The gas space was filled with a mixture of 95 per cent nitrogen and 5 per cent carbon dioxide. The final volume was made up to 3.0 cc.; bath temperature 38°.

Oxidation of Fatty Acids by Liver

Most of our experiments have been carried out with the enzyme system prepared from rabbit kidney, but the salient features of fatty acid oxidation as described above have been found also to apply to the system prepared from rabbit liver. Here also the oxidation of fatty acids has to be sparked by some member of the citric acid cycle and this sparked oxidation, under suitable conditions, proceeds to completion; *i.e.*, to carbon dioxide and water by way of the citric acid cycle (Table IV). The liver fatty acid oxidase system is somewhat less stable than the kidney system, but, providing care is taken to maintain the pH during homogenization, there is not much choice between the two systems.

TABLE IV

Oxidation of Fatty Acids in Rabbit Liver System

Details as in Table I, except for the addition of 5 micromoles of α-ketoglutaric acid as the sparker in both the control and the experimental cups. The observed oxygen uptake includes the extrapolation correction for the first 5 minutes, during which time the stop-cocks were not closed.

Fatty acid	Oxygen uptake in microatoms			Theory for oxidation to isobutyric acid
	Micromoles added	Observed	Theory for complete oxidation	
Isocrotonic	5	38	45	
Butyric	5	53	50	
	5	51.5	50	
Caproic	3	42.5	48	
trans-$\Delta^{\alpha,\beta}$-Hexenoic	3	39	45	
Sorbic	5	63	70	
	7.5	105	105	
Isocaproic	5	32.7		30
Heptylic	2.5	26.2		30
Caprylic	5	77	110	
β-Hydroxycaprylic	5	72	105	

The liver system differs from the corresponding system in kidney in several respects. (*a*) It contains enzymes capable of oxidizing propionic or phenylpropionic acid. The oxidation of propionic acid proceeds to completion. (*b*) The oxidation of isocaproic acid in liver[6] proceeds to completion, *i.e.* to carbon dioxide and water, and, consistent with that observation, it can be shown that isobutyric acid is likewise oxidized to completion. By contrast, the kidney system carries the oxidation of isocaproic acid only as far as the stage of isobutyric acid (or propionic acid). (*c*) Acetic and acetoacetic acids are barely, if at all, oxidized by liver preparations at the stage of the 3rd residue, although these substances are oxidized readily in the kidney system. It should be pointed out that the inability to oxidize acetic acid is related to the problem of activating acetic acid and not to the problem of whether activated acetic acid is formed during fatty acid oxidation. (*d*) The liver system has lent itself more readily to the demonstration of the "intermediates," particularly acetoacetic acid, and this property may be interpreted in terms of a disproportion between the amount of fatty acid oxidizing enzymes and the amount of the cyclophorase system. (*e*) The liver fatty acid system at the stage of the 3rd residue is often not complete when fortified with the usual components. Some factor which is other than the sparker or adenylic acid, and which is present in an aqueous extract of boiled heart muscle, is needed to restore fully the activity of the liver system.

---

[6] The propionic, phenylpropionic, and isobutyric oxidases can be demonstrated in liver preparations only at the stage of the 1st or 2nd residues (R_1L, or R_2L). Apparently some factor present in the first discarded supernatant is either essential for these oxidases or is important in their stabilization.

Mechanisms of Fatty Acid Oxidation in Liver and Kidney

The data presented thus far have thrown little light on the mechanism of oxidative degradation of fatty acids, primarily because they deal with conditions under which fatty acid oxidation proceeds to completion, and which, therefore, do not lend themselves to the isolation and demonstration of the "intermediates." Some data are available which bear on the question of mechanism and, while the documentation is still incomplete, certain essential features of mechanism are already recognizable. For reasons of simplicity it may be preferable to list separately the events involved in the oxidation respectively of caproic and valeric (or isocaproic) acids.

Caproic acid	*Valeric acid (or isocaproic acid)*
1. β oxidation to β-ketocaproic acid by way of either α,β-unsaturated or β-hydroxy acid	1. β oxidation to β-ketovaleric acid (or β-ketoisocaproic acid) by way of either α,β-unsaturated or β-hydroxy acid
2. Cleavage of β-ketocaproic acid to acetic and butyric acids	2. Cleavage of β-ketovaleric acid (or β-ketoisocaproic acid) to acetic and propionic acids (or isobutyric acid)
3. Condensation of acetic and oxalacetic acids to form citric acid	3. Condensation of acetic and oxalacetic acids to form citric acid
4. β oxidation of butyric to acetoacetic acid	4. Complete oxidation of citric acid in cyclophorase system
5. Condensation of acetoacetic acid with oxalacetic acid to form citric acid	
6. Complete oxidation of citric acid in cyclophorase system	

The oxidation of caproic acid differs from that of valeric acid or isocaproic acid in two respects: (*a*) it leaves no residue after oxidation and (*b*) it gives rise to acetoacetic acid. The reactions of acetoacetic acid will be considered separately from those of other β-keto acids, although the mechanisms may well be identical.

TABLE V

Acetoacetic Acid Formation during Fatty Acid Oxidation in Liver

Each of the fatty acids was tested in the presence of 1.5 cc. of liver enzyme at the R_1 residue stage, 0.6 cc. of 1 per cent heart extract, and 0.3 cc. of 0.01 M α-ketoglutarate. Final volume 3 cc., oxygen in the gas space 38°. The value for acetoacetic acid found at the end of the experiment in the blank was usually about 1 micromole or less.

Experiment No.	Fatty acid	Micromoles added	Microatoms oxygen absorbed (corrected for blank)	Micromoles acetoacetic acid formed (corrected for blank)
1	Caproic	40	163	14.8
	β-Hydroxycaproic	40	124	13.1
	cis-$\Delta^{1,2}$-Hexenoic	40	30	1.0
	trans-$\Delta^{1,2}$-Hexenoic	40	119	15.1
	Sorbic	40	84	12.5
2	Butyric	50	40	5.4
	Crotonic	50	21	6.6
	Isocrotonic	50	34	6.6
	Vinylacetic	50	32	7.2
	β-Hydroxybutyric	50	22	13.6
3	Caproic	40	154	15.0
	Octanoic	20	132	12.8
	Valeric	60	79	2.1
	Heptylic	30	114	3.8
	Isocaproic	60	93	1.4
4	Caproic	40	163	14.8
	Valeric	60	99	0.4
	α,β-Pentenoic	60	75	0.3
	Isocaproic	60	80	1.1
	4-Methyl-$\Delta^{1,2}$-pentenoic	60	105	0.4

Evidence for β Oxidation—The results of Table II show conclusively that all the postulated "intermediates" formed during β oxidation of fatty acids in the kidney system are oxidized to the same products and at approximately the same speed as the parent fatty acids. By contrast, no α- or γ-substituted fatty acids were found to undergo oxidation. The liver system provides further confirmation of β oxidation. Under the conditions obtaining in Experiments 1 to 4 summarized in Table V, even numbered fatty acids from C_4 to C_8 are found to give rise to considerable amounts of acetoacetic acid. Significantly, the postulated "intermediates" of β oxidation of these fatty acids give rise to approximately the same amount of acetoacetic acid as the parent fatty acids. It is to be noted that the odd numbered fatty acids or isocaproic acid, although oxidized readily under the same experimental conditions, give rise to little if any acetoacetic acid.[7] Thus acetoacetic acid in this series of experiments must be presumed to arise exclusively from butyric acid as an intermediate and the conclusion is permissible that caproic and octanoic acids as well as their corresponding products of β oxidation are oxidized to acetoacetic acid via butyric acid as an "intermediate."

The above evidence is only indirect in that it does not establish the formation of β-hydroxy, α,β-unsaturated acids, or β-keto acids as "intermediates" of β oxidation, but merely shows that these derivatives behave as true "intermediates." Acetoacetic acid is the one "intermediate" of β oxidation which has been established by direct means.

TABLE VI

Oxidation of β-Hydroxy Acids by Liver System

Acid	Microatoms oxygen taken up (blank subtracted)	β-Keto acid formed	Micromoles keto acid formed (blank subtracted)
dl-β-Hydroxybutyrate	38.2	Acetoacetic	21.1
dl-β-Hydroxyvalerate	22.8	β-Ketovaleric	15.1
dl-β-Hydroxycaproate	24.0	Acetoacetic	3.9
dl-β-Hydroxyoctanoate	19.7	"	3.4
dl-β-Hydroxyisocaproate	0.3		0.6

Each manometer cup contained 1.5 cc. of rabbit liver enzyme at the 3rd residue stage, 0.2 cc. of 0.01 M adenylic acid, 0.1 cc. of 0.125 M phosphate buffer of pH 7.2, 0.1 cc. of 0.02 M magnesium sulfate, and 1 cc. of 0.1 M *dl*-β-hydroxy acid. Final volume 3.0 cc., alkali in the center well, 38°.

There is present in both the liver and kidney fatty acid oxidizing systems an enzyme which catalyzes the oxidation of β-hydroxybutyric acid and some of its higher homologues to the corresponding keto acids. This oxidase is unique among the fatty acid oxidizing enzymes in that its substrate does not have to be activated. To what extent this enzyme participates in fatty acid oxidation is uncertain, but at any rate it provides the most direct demonstration of one phase of β oxidation. The data of Table VI show the action of the β-hydroxy acid oxidase of the liver system on several β-hydroxy acids. It will be noted that no sparker has to be provided for these oxidations to proceed. Only in the case of β-hydroxyvalerate does the ratio, microatoms of oxygen absorbed to micromoles of keto acid formed, approach the theoretical value of 1. In some experiments the theoretical values for β-hydroxyvalerate and butyrate have indeed been attained, but under the same conditions the yield of keto acid resulting from the oxidation of β-hydroxycaproate and β-hydroxyoctanoate has been almost negligible. This would suggest that in the latter two cases oxidation proceeds beyond the keto acid stage. As a matter of fact the corresponding keto acids are not formed, but instead acetoacetic acid[8] accumulates. No success has attended efforts to stop the oxidation of β-hydroxycaproate and β-hydroxyoctanoate at the stage of the corresponding β-keto acids. The only β-keto acids which appear to be stable under the conditions of the experiment are acetoacetic acid and β-ketovaleric acid. Coincident with the oxidation of hydroxy acids there is esterification of inorganic phosphate and it may well be this circumstance which explains why the β-keto acids formed in the oxidation undergo further transformation, even though added β-ketohexanoic and β-ketooctanoic acids are immune to oxidation under the same conditions. The β-hydroxy acid oxidase appears to be a self-sparking system like the oxidase which oxidizes pyruvic acid to acetoacetic acid.

---

[7] The fact that odd numbered fatty acids do not give rise to acetoacetic acid makes it necessary to distinguish this rabbit liver system from the rat liver system of Lehninger (9) in which acetoacetic acid rises equally well from odd as from even numbered fatty acids. Acetoacetic acid accumulates in the crude rabbit liver system apparently because of the presence of some substance or substances which inhibit the subsequent oxidation of acetoacetic acid. In more purified rabbit liver systems, acetoacetic acid fails to accumulate to any significant amount.

The oxidation of the β-hydroxy acid to the keto acid is a reversible one. As shown in Table VII, the reduction of various β-keto acids can be linked with the oxidation of α-ketoglutaric acid to succinic acid and carbon dioxide. The oxidation-reduction reaction can be followed manometrically by measuring carbon dioxide formation. The reversible nature of the hydroxy acid oxidase system can also be demonstrated with the oxidation-reduction indicator, benzyl viologen. In the presence of β-hydroxybutyrate and under anaerobic conditions, the indicator is reduced to the blue semiquinoid form, whereas, when acetoacetate is introduced into the enzyme mixture from a side arm, the blue color is rapidly discharged.

Cleavage of β-Keto Acids to Acetic Acid and Residue Fatty Acid—This phase of fatty acid oxidation is now readily demonstrable. Valeric acid

---

[8] The rate with which β-keto acids are catalytically decomposed by aniline varies inversely with the length of the carbon chain. By carrying out the rate studies under standard conditions it becomes possible to determine whether acetoacetic acid or a higher β-keto acid is present in the medium. The method is applicable or reliable when only one β-keto acid is present. Rate studies applied to the identification of the β-keto acids which accumulated during the oxidation of β-hydroxycaproic and octanoic acids showed unequivocally that the curves were superimposable with that of acetoacetic acid and deviated very widely from those of the higher β-keto acids. However, the formation of β-ketovalerate from β-hydroxyvalerate was confirmed by the analysis of the curve for catalytic decomposition.

(or β-ketovaleric acid) on oxidation gives rise to propionic acid, which has been identified and characterized by the counter-current distribution method. Similarly, isocaproic acid (or β-ketoisocaproic acid) gives rise to isobutyric acid. These two end-products could arise only as the result of a cleavage reaction leading to the formation of acetic acid. In that connection the inactivity of isovaleric acid is significant and isovaleric acid can be excluded as an "intermediate" in the oxidation of isocaproic to isobutyric acid.

The cleavage of β-keto acids into acetic acid is best shown by experiments in which acetic acid is trapped, so to speak, by condensation with oxalacetic acid. The extra formation of citric acid from oxalacetic acid in the presence of β-keto acids (Tables VIII and IX) generally provides the most direct evidence that acetic acid arises during the breakdown of β-keto acids. These results are in agreement with the experiments reported by Breusch (24), Wieland and Rosenthal (25), and Hunter and Leloir (23). This Breusch effect (17) might be interpreted in terms of the β-keto acids acting as hydrogen acceptors. However, this interpretation can be ruled out, since (*a*) the experiments are carried out in oxygen, (*b*) oxygen is needed for the reaction, and (*c*), as shown in Table IX, β-hydroxybutyric acid can replace acetoacetic acid quantitatively in this reaction.

TABLE VII

Reduction of β-Keto Acids by α-Ketoglutaric Acid

Addition	Microliters CO_2 in 30 min.
α-Ketoglutarate	0
" + acetoacetate	111
" + β-ketovalerate	93
" + β-ketohexanoate	85
" + β-ketoisocaproate	44
" + β-ketooctanoate	3
Acetoacetate (no α-ketoglutarate)	0

Each manometer cup contained 1.5 cc. of kidney enzyme at the R_3 residue stage, 0.3 cc. of 0.01 M adenylic acid, 0.2 cc. of 0.02 M magnesium phosphate, 0.2 cc. of 0.125 M phosphate buffer of pH 7.3, 0.1 cc. of 0.5 M sodium bicarbonate, 0.3 cc. of 0.1 M α-ketoglutarate, and 0.5 cc. of 0.1 M *dl*-β-hydroxy acid. 95 per cent nitrogen, 5 per cent carbon dioxide gas mixture in the air space.

Condensation of Acetic Acid and Oxalacetic Acid to Form Citric Acid—It can readily be demonstrated that acetic acid is not directly oxidized in the kidney enzyme system. Every conceivable stable "intermediate" such as glycolic acid, oxalic acid, glyoxylic acid, and glycolaldehyde has been tested and found to be inactive. The alternative to direct oxidation is, of course, by way of condensation with oxalacetic acid and then by entry into the reactions of the cyclophorase system.

The use of isotopes has been essential to decide whether acetic acid can condense with oxalacetic acid to form citric acid. The isotope experiments[9] will be presented in a later communication.

β Oxidation of Butyric Acid to Acetoacetic Acid—The data of Table IV establish the formation of acetoacetic acid from butyric acid or from fatty acids which give rise to butyric acid.

TABLE VIII

Citric Acid Formation from Condensation of Oxalacetic and Acetoacetic Acids

The system contained 1 cc. of enzyme at the R_3K residue stage, 0.05 cc. of M barium chloride, and 0.1 cc. of 0.02 M magnesium sulfate. Final volume, 3.0 cc. Experiment carried out in air at 38° for 1 hour. Citric acid was estimated by the method of Pucher *et al.* (22) as modified by Hunter and Leloir (23). Every estimation of an experimental solution was carried out with a parallel estimation of the boiled enzyme control for that particular solution. The above results are already corrected for the "citric acid" found in the control solutions with boiled enzyme. The control values never exceeded 1 micromole of citric acid.

The results are expressed in micromoles.

System with	Experiment 1. 70 micromoles oxalacetic, 30 micromoles acetoacetic	Experiment 2. 60 micromoles oxalacetic, 30 micromoles acetoacetic	Experiment 3. 40 micromoles oxalacetic, 25 micromoles acetoacetic	Experiment 4. 40 micromoles oxalacetic, 25 micromoles acetoacetic	Experiment 5. 70 micromoles oxalacetic, 30 micromoles acetoacetic
No additions	0.4	0.2	0.2	0.3	0.3
Oxalacetic acid	1.2	5.3	3.8	3.5	2.6
" and acetoacetic acids	7.9	10.3	11.2	9.2	11.6
Acetoacetic acid	0.4			0.4	0.3

Condensation of Acetoacetic Acid and Oxalacetic Acid to Form Citric Acid—Table VIII has already summarized the evidence on this point. The interaction of acetoacetic and oxalacetic acids may be considered to be the result of two separate reactions: (1) cleavage of activated acetoacetic into 2 molecules of acetic acid, and (2) condensation of activated acetic acid with oxalacetic acid. Acetoacetic acid does not give rise to acetic acid unless activated.

The yield of citric acid from a mixture of oxalacetic and acetoacetic acid is usually at least double that from oxalacetic acid alone, whereas the yield from a mixture of oxalacetic and acetic acid is rarely greater than that from oxalacetic acid alone.

Complete Oxidation of Citric Acid in Cyclophorase System—In our previous communication the complete oxidation of citric acid or of any of its related forms to carbon dioxide and water has already been established for the kidney cyclophorase system. The conditions which favor the preparation of an active cyclophorase system are identical with those which favor the preparation of active fatty acid systems. Both the cyclophorase system and the fatty acid oxidase system require magnesium ions, adenylic acid (or adenosine triphosphate), and phosphate ions for maximal activity. The main distinction is that the cyclophorase system is complete when provided with any member of the citric acid cycle and the above components. The fatty acid system does not start to function under the same conditions unless the cyclophorase system is also functioning initially.

---

[9] Knox, W. E., and Weinhouse, S., unpublished experiments.

Table IX

Citric Acid Formation from Oxalacetic Acid and β-Keto Acids

Additions	Micromoles citric acid formed	Additions	Micromoles citric acid formed
None	0.2	None	0.2
Oxalacetic	6.3	Oxalacetic	4.9
" + β-ketovaleric	10.3	" + acetoacetic	11.3
β-Ketovaleric	0.4	" + β-hydroxybutyric	10.4
Oxalacetic + β-hydroxybutyric	10.0	Oxalacetic + β-ketovaleric	9.1
β-Hydroxybutyric	0.2	" + β-ketohexanoic	9.2
		Oxalacetic + β-ketooctanoic	5.8

Each manometer cup contained 1.5 cc. of kidney enzyme at the 3rd residue stage, 0.3 cc. of 0.01 M adenylic acid, 0.2 cc. of magnesium sulfate, 0.3 cc. of 0.125 M phosphate buffer of pH 7.2, and 0.1 cc. of M barium chloride. Where indicated, 0.6 cc. of 0.1 M oxalacetate and 0.6 cc. of 0.1 M β-keto acid or hydroxy acid were used. The experiments were carried out in oxygen gas at 38°.

Activation of Fatty Acids and "Intermediates"—The discussion of the mechanism of fatty acid oxidation in the liver and kidney systems would be incomplete without reference to the problem and nature of the so called sparking or activation phenomenon. Another communication will be reserved for the systematic consideration of oxidative phosphorylation and the sparking phenomenon. For present purposes it will be sufficient to state that fatty acids and their products of oxidation are not oxidized as such but only after combination with what appear to be pyrophosphate-containing coenzymes as yet unspecified.

EXPERIMENTAL

Enzyme suspensions were prepared from rabbit kidneys as previously described, with the addition of alkali during homogenization with 0.9 per cent potassium chloride (1). Unless otherwise specified, a suspension in dilute salt solution of the 3rd residue, designated R_3K (the residue from centrifugation of the homogenate twice resuspended and washed with potassium chloride), was used in all the experiments, which were performed in Warburg manometers at 38°. The enzyme (1, 1.5, or 2 cc. per cup) was routinely supplemented by the following additions: 0.3 cc. of 0.01 M adenosine triphosphate, 0.2 cc. of 0.02 M magnesium sulfate, and 0.5 cc. of 0.04 M phosphate buffer of pH 7.3, and in most instances by the further addition of 0.3 cc. of a 1 per cent aqueous extract of acetone powder of pig heart, acidified to about pH 6 with HCl (for preparation, see Knox *et al.* (11)). A gas phase of 100 per cent O_2 was used, as this was found to increase oxidative rates markedly above those observed in air. Controls without added substrate were included in all experiments. All the substrates were added in solution as neutral sodium salts.

Butyric, valeric, isovaleric, capric, lauric, myristic, phenylpropionic, and cinnamic acids were obtained from the Eastman Kodak Company. Pure samples of *n*-caproic, *n*-caprylic, stearic, oleic, and azelaic acids were generously provided by Dr. David Rittenberg. The Armour Laboratories kindly provided *n*-heptylic, *n*-undecylic, and *n*-tridecylic acids. *trans*-$\Delta^{\alpha,\beta}$-Hexenoic and styrylacrylic acids were the gifts of the Carbide and Carbon Chemicals Corporation. The Farchan Laboratories prepared for us highly purified and authentic samples of phenyloctanoic, phenylcapric, phenyllauric, acrylic, 3,4-epoxybutyric, *cis*-$\Delta^{\alpha,\beta}$-hexenoic, isocrotonic, $\Delta^{2,3}$-pentenoic, $\Delta^{4,5}$-pentenoic, dimethylacrylic, and 4-methylpentenoic acids; also the ethyl esters of β-hydroxyvaleric, β-hydroxyhexanoic, β-hydroxyisocaproic, β-hydroxyoctanoic, β-hydroxylauric, β-ketoisocaproic, and β-ketooctanoic acids. Dr. Herbert E. Carter generously provided us with samples of various alkyl- and phenyl-substituted fatty acids, with the "intermediate" for the preparation of phenylvaleric acid, and with a pure sample of *dl*-threo-2,3-dihydroxybutyric acid.

Acetoacetic acid was prepared by hydrolysis of the ethyl ester with the theoretical quantity of alkali, followed by the removal of ethyl alcohol by vacuum distillation. The same procedure was applied to the preparation of the other β-keto and β-hydroxy acids from their respective ethyl esters. *dl*-β-Hydroxybutyric and *dl*-β-hydroxycaproic acids were prepared from the corresponding keto acids by reduction with sodium amalgam in alkaline solution. γ-Hydroxybutyric acid (γ-butyrolactone), succinic semialdehyde, and vinylacetic acids were prepared by the methods of Sircar (26), Dakin (27), and Falaise and Frognier (28) respectively. Vinylacetic acid was purified according to Rietz (29). β-Benzoylpropionic and γ-phenylbutyric acids were synthesized by the methods given in "Organic syntheses" (30). β-Ketovaleric and β-ketocaproic ethyl esters were prepared according to the method of Fischer *et al.* (31).

All the keto acids tested were estimated manometrically as carbon dioxide by the aniline-citrate manometric method (20). Succinic semialdehyde was estimated by the bisulfite method of Clift and Cook (32).

SUMMARY

The complete oxidation of fatty acids and their derivatives to carbon dioxide and water has been studied in kidney and liver cyclophorase preparations.

The expenses of this investigation were defrayed by grants from the American Cancer Society, the Rockefeller Foundation, and the Williams-Waterman Fund of the Research Corporation.

BIBLIOGRAPHY

1. Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, **172**, 389 (1948).
2. Leloir, L. F., and Muñoz, J. M., *Biochem. J.*, **33**, 734 (1939).
3. Muñoz, J. M., and Leloir, L. F., *J. Biol. Chem.*, **147**, 355 (1943).
4. Leloir, L. F., and Muñoz, J. M., *J. Biol. Chem.*, **153**, 53 (1944).
5. Lehninger, A. L., *J. Biol. Chem.*, **154**, 309 (1944).
6. Lehninger, A. L., *J. Biol. Chem.*, **157**, 363 (1945).
7. Lehninger, A. L., *J. Biol. Chem.*, **161**, 437 (1945).
8. Lehninger, A. L., *J. Biol. Chem.*, **161**, 413 (1945).
9. Lehninger, A. L., *J. Biol. Chem.*, **164**, 291 (1946).
10. Lehninger, A. L., *J. Biol. Chem.*, **165**, 131 (1946).
11. Knox, W. E., Noyce, B. N., and Auerbach, V. H., *J. Biol. Chem.*, **176**, 117 (1948).
12. Straub, B., *Z. physiol. Chem.*, **244**, 117 (1936).
13. Atchley, W. A., *J. Biol. Chem.*, **176**, 123 (1948).
14. Knoop, F., *Beitr. chem. Physiol. u. Path.*, **6**, 150 (1904).
15. Stadie, W. C., *Physiol. Rev.*, **25**, 395 (1945).
16. Breusch, F. L., *Science*, **97**, 490 (1943).
17. Breusch, F. L., *Enzymologia*, **11**, 169 (1944).
18. Breusch, F. L., and Keskin, H., *Enzymologia*, **11**, 243 (1944).
19. Wood, H. G., *Physiol. Rev.*, **26**, 198 (1946).
20. Ostern, P., *Z. Physiol. Chem.*, **218**, 160 (1933). Edson, N. L., *Biochem. J.*, **29**, 2082 (1935).
21. Bartlett, G. R., and Barron, E. S. G., *J. Biol. Chem.*, **170**, 67 (1947). Kalnitsky, G., and Barron, E. S. G., *J. Biol. Chem.*, **170**, 83 (1947).
22. Pucher, G. W., Wakeman, A. J., and Vickery, H. B., *Ind. and Eng. Chem., Anal. Ed.*, **13**, 244 (1941).
23. Hunter, F. E., and Leloir, L. F., *J. Biol. Chem.*, **159**, 295 (1945).
24. Breusch, F. L., *Biochem. J.*, **33**, 1757 (1939).
25. Wieland, H., and Rosenthal, C., *Ann. Chem.*, **554**, 241 (1943).
26. Sircar, S. S. G., *J. Chem. Soc.*, **898** (1928).
27. Dakin, H. D., *Biochem. J.*, **11**, 79 (1917).
28. Falaise, L., and Frognier, R., *Bull. Soc. chim. Belg.*, **42**, 427 (1933).
29. Rietz, E., in "Organic syntheses," New York, **24**, 96 (1944).
30. Somerville, L. F., and Allen, C. F. H., in Organic syntheses, New York, 2nd edition, coll. **2**, 81 (1944); Martin, E. L., in Organic syntheses, New York, 2nd edition, coll. **2**, 499 (1944).
31. Fischer, H., Goldschmidt, M., and Nussler, W., *Ann. Chem.*, **486**, 1 (1931).
32. Clift, F. P., and Cook, R. P., *Biochem. J.*, **26**, 1788 (1932).

STUDIES ON THE CYCLOPHORASE SYSTEM

III. OBLIGATORY SPARKING OF FATTY ACID OXIDATION

By W. EUGENE KNOX,* B. N. NOYCE, and V. H. AUERBACH

(*From the Enzyme Laboratory, Department of Medicine, College of Physicians and Surgeons, Columbia University, New York*)

(Received for publication, April 28, 1948)

The fatty acid oxidizing system, prepared from washed rabbit kidney as described in the previous paper (1), often did not oxidize fatty acids unless supplemented by a small amount of one of the cyclophorase substrates. A similar effect could be obtained by adding an extract of boiled heart muscle. This addition was regularly found to be necessary for fatty acid oxidation by an enzyme at the 3rd residue (R_3) stage which had been prepared with precautions to insure thorough washing at each centrifugation. The effects of fumarate or of heart extract addition on such an enzyme are comparable (Fig. 1). Virtually no oxidation of butyrate occurs in the absence of any addition, whereas a maximal rate of butyrate oxidation is achieved by the addition of an amount of either substrate or heart extract which in the absence of butyrate is responsible for an insignificant oxygen uptake. Suboptimal additions of either produce proportionally lower rates of butyrate oxidation. Other substrates directly oxidizable by the cyclophorase system, when tested in comparable concentrations, were found to produce the same effect as fumarate and heart extract.

All the known coenzymes were assayed in this system for activity in initiating oxidation of butyrate.[1] The slight activity, found in several of the preparations which were not available in pure form, disappeared upon further purification. Only glutathione was active, producing an effect equal to that of fumarate or glutamate on a molar basis (Table I). The glutamic acid moiety of glutathione is oxidized by the cyclophorase system. Analysis of a sample of heart extract[2] showed 0.05 micromole of glutathione

---

* The expenses of this investigation were defrayed by grants from the American Cancer Society, the Rockefeller Foundation, and the Williams-Waterman Fund of the Research Corporation.

Present address, Northwestern University, the Rheumatic Fever Research Institute, Chicago, Illinois.

[1] The following compounds were tested: coenzyme I, coenzyme II, flavin-adenine dinucleotide, riboflavin monophosphate, cytochrome *c*, adenosine triphosphate (ATP), adenosine-3-phosphate, thiamine pyrophosphate, pyridoxal phosphate, glutathione, coenzyme of acetylation (from Dr. David Nachmansohn), vitamin B_c conjugate, and pteroylglutamic acid.

[2] The heart extract was prepared as follows: 1 kilo of fresh pig heart was trimmed, minced in the cold, and extracted after 10 minutes with 2 volumes of water at 95–100°

per mg. of dry weight of extract, determined iodometrically (2), and 0.16 micromole per mg. of succinate plus glutamate, determined enzymically

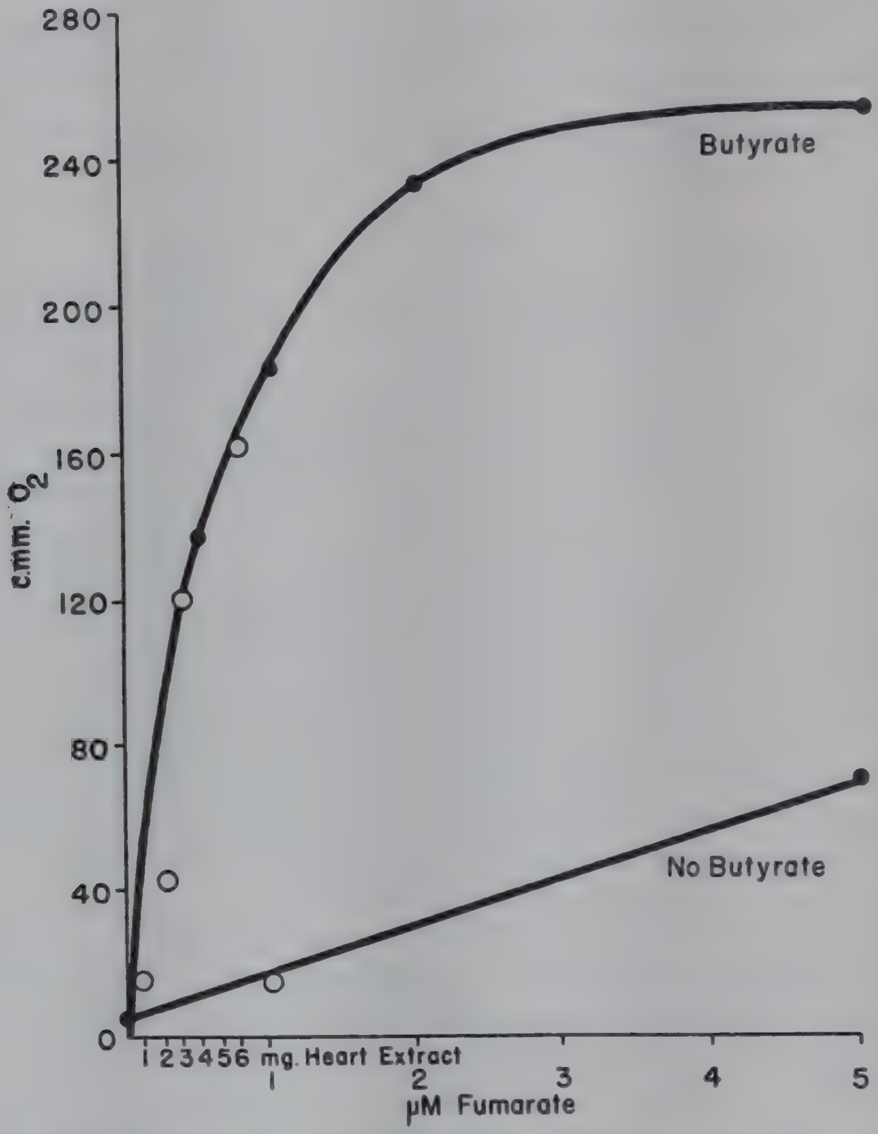


Fig. 1. Sparking of butyrate oxidation by fumarate and by heart extract. Each cup contained 1 cc. of kidney enzyme at the 3rd residue stage (R_3) 3½ hours old, 0.3 cc. of 0.01 M ATP, 0.2 cc. of 0.02 M $MgSO_4$, 0.5 cc. of 0.04 M phosphate buffer, pH 7.25; total volume 3.0 cc.; O_2 in the gas phase, NaOH in the center well; equilibrated at 38° for 7 minutes before closing the taps. Fumarate (●) or heart extract (○) in the amounts shown, and 0.3 cc. of 0.1 M butyrate where indicated, were added to the cups beforehand. The oxygen uptake was recorded during the first 10 minutes after equilibration.

after chloramine-T oxidation (3). This heart extract, which thus contained at least 0.21 micromole per mg. of oxidizable substrates, had the same effect

---

The aqueous extract after chilling was precipitated with 4 volumes of acetone and the precipitate dried with acetone and ether.

per mg. on butyrate oxidation as 0.25 micromole of substrate. Most, if not all, of the effect of the heart extract can therefore be attributed to the presence of traces of these three substrates directly oxidizable by the cyclophorase system.

TABLE I

Sparking of Butyrate Oxidation by Fumarate, Glutamate, and Glutathione

Same conditions as in the legend of Fig. 1. One enzyme preparation was used in the comparison of fumarate with glutamate and another preparation for the comparison of fumarate with glutathione.

Addition	Amount	O_2 per 5 min.	Addition	Amount	O_2 per 10 min.
	micromole	*c.mm.*		*micromole*	*c.mm.*
		8			12
Fumarate	0.4	29	Fumarate	0.2	30
Glutamate	0.4	18	Glutathione	0.2	19
Fumarate	0.8	93	Fumarate	0.4	84
Glutamate	0.8	90	Glutathione	0.4	82
Fumarate without butyrate	0.8	7	Fumarate	0.8	107
Glutamate without butyrate	0.8	8	Glutathione	0.8	126
			Fumarate without butyrate	0.8	10
			Glutathione without butyrate	0.8	10

TABLE II

Comparison of Amount of Fumarate Necessary to Initiate Oxidation of Some Fatty Acids

The oxygen uptake, without the subtraction of the blanks, is given for the fatty acids alone and with added fumarate, and was determined under the conditions given in the legend of Fig. 1.

Fatty acid added	C.mm. O_2 per 15 min.		
	Fatty acid alone	+ 0.5 micromole fumarate	+ 3.0 micromole fumarate
Butyrate, 30 micromoles	25	218	347
Octanoate, 5 "	12	158	
" 20 "	13	24	174
Acetate, 30 micromoles	20	28	151
Acetoacetate, 30 micromoles	17	28	83
No fatty acid	15	18	60

Amount of Cyclophorase Substrate Required to Initiate Oxidation of Different Fatty Acids—In the initial phase of these investigations, two types of oxidative reactions involving fatty acids were distinguished, depending upon whether or not additional cyclophorase substrate was required. However, even in the case in which no substrate had to be added, the test system contained heart extract, which served as a minimal source of substrate. The difference originally observed between various fatty acids is one of degree. All require some substrate to initiate their oxidation, but some fatty acids require more than others (Table II). These necessary amounts of substrate may be added directly or in the form of heart extract, or may already be present in the trace amounts required for certain reactions in less well washed enzyme preparations. By controlling these factors, it has been possible to show that oxidation of all the fatty acids and fatty acid derivatives which we have studied must be sparked by the simultaneous oxidation of a small amount of a cyclophorase substrate. There is no evidence that an unsparked oxidation of fatty acids can occur. The amount required is characteristic of the conditions used and of the fatty acid oxidized, and bears no relation to the total amount of fatty acid that can be oxidized once the reaction begins.

In consequence of this obligatory nature of the sparking reaction, the fact that butyrate and octanoate can frequently be oxidized to completion under conditions in which added acetoacetate is not oxidizable is no bar to accepting acetoacetate as an intermediate. All three compounds require sparking, though in different amounts, and all three compounds, when changed by the sparking reaction, may share a common pathway.

Nature of Sparking Reaction

All attempts to initiate the oxidation of fatty acids in some way not involving simultaneous oxidation of a cyclophorase substrate have been unsuccessful. There is no evidence, for example, that an initial anaerobic condensation occurs between the fatty acid and pyruvate or oxalacetate. Although added $NaHCO_3$ will often increase the blank oxygen uptake of enzyme at the 3rd residue stage (R_3) and initiate fatty acid oxidation, this is due to the presence in the enzyme of a small amount of pyruvate, which with the added CO_2 can be oxidized through its conversion to oxalacetate (4). Further washing to remove these traces of pyruvate prevents this artifact of "sparking with CO_2" (Table III). As would be expected, such a pyruvate-free enzyme then requires a larger amount of substrate to initiate fatty acid oxidation. Finally, none of the many fatty acid derivatives tested can be oxidized directly without sparking. These cannot therefore be identical with the intermediates produced by the sparking reaction.

In the previous communication (1) the use of ferricyanide as an oxidizing agent for fatty acid oxidation was described. It was of considerable interest to determine whether the sparking phenomenon would apply when ferricyanide replaced oxygen as oxidizing agent. The data of Table IV establish that the sparking phenomenon is concerned with the primary dehydrogenation of the cyclophorase substrate, and not with the subsequent hydrogen transport through the systems reacting with oxygen.

The initiation of fatty acid oxidation is obligatorily associated with the simultaneous oxidation of a small amount of a cyclophorase substrate. The oxidations of the several substrates which can spark fatty acid oxidation all produce esterification of phosphate. Of particular interest is the effect of dinitrophenol and gramicidin on these oxidations. Dr. J. V. Taggart and his colleagues have shown that these two reagents prevent esterification of inorganic phosphate without inhibiting the oxidation of the cyclophorase substrates. They also completely prevent fatty acid oxidation even when a substrate of the cyclophorase system is undergoing rapid oxidation. However, the energetic coupling of the initial fatty acid reaction with the oxidation of the cyclophorase substrates suggested by these observations cannot be attributed simply to the generation of ATP (5). ATP, in an amount greater than can be destroyed during the experiment, does not

Table III

Effect of Added CO_2 on Butyrate Oxidation by Kidney Enzyme Preparations at 3rd and 5th Residue Stages

0.2 cc. of 0.1 M $NaHCO_3$ was added as indicated. The conditions are otherwise as in the legend for Fig. 1. The values are c.mm. of O_2 per 15 minutes.

Enzyme	CO_2 only	Butyrate only	CO_2 + butyrate	0.6 micromole fumarate only	Butyrate + 0.6 micromole fumarate	Butyrate + 3.0 micromoles fumarate
R_3K	25	19	165	12	114	
R_5K	17	17	23	13	12	194

Table IV

Sparking of Fatty Acid Oxidation in System containing Ferricyanide As Oxidizing Agent

Each cup contained 1.5 cc. of kidney enzyme at the 3rd residue stage, 0.2 cc. of 0.02 M magnesium sulfate, 0.3 cc. of 0.01 M adenesine triphosphate, 0.1 cc. of 0.5 M sodium bicarbonate, and 0.2 cc. of 0.5 M sodium ferricyanide. Total volume, 3.0 cc. Gas space filled with 95 per cent nitrogen-5 per cent carbon dioxide.

Additions	C.mm. CO_2, 1st 10 min.	C.mm. CO_2, 2nd 10 min.
None	6	4
α-Ketoglutarate (2 micromoles)	59	45
Butyrate (10 micromoles)	40	21
" (10 ") + α-ketoglutarate (2 micromoles)	272	241
cis-Hexenoate (10 micromoles)	41	24
" (10 ") + α-ketoglutarate (2 micromoles)	226	218

replace an oxidizable substrate as the sparker. Similarly, the acyl phosphates of the fatty acids, which have been suggested as the active form for oxidation (5, 6), cannot be oxidized in this system without sparking. During the sparked oxidation of a fatty acid, no acyl phosphate accumulates that can be determined by Lipmann and Tuttle's hydroxylamine reaction (7).

The initial reaction of a fatty acid has therefore not yet been dissociated from the simultaneous oxidation of a directly oxidizable substrate. Another function of this added substrate, in addition to sparking, must also be that of condensing with activated acetate and acetoacetate formed during fatty acid oxidation. By this condensation fatty acids may be oxidized to completion through the citric acid cycle, as will be shown by isotopic experiments to be published later. A reaction analogous to this, the condensation of acetoacetate with oxalacetate to form citric acid, has already been demonstrated in this system (1).

SUMMARY

Oxidation of fatty acids and their derivatives by the enzymes from rabbit kidney can occur only if a small amount of cyclophorase substrate is first oxidized to initiate the reaction. This sparking effect by compounds of the citric acid cycle consists of a primary activation of the fatty acid which is not duplicated by ATP or acyl phosphates, and of a later condensation between a cyclophorase substrate and the "acetate" leading to complete oxidation through the cycle.

BIBLIOGRAPHY

1. Grafflin, A. L., and Green, D. E., *J. Biol. Chem.*, **176**, 95 (1948).
2. Sabalitschka, T., in Bamann, E., and Myrbäck, K., Methoden der Fermentforschung, New York, 428 (1941).
3. Cohen, P. P., *Biochem. J.*, **33**, 551 (1939).
4. Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, **172**, 389 (1948).
5. Lehninger, A. L., *J. Biol. Chem.*, **157**, 363 (1945).
6. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **161**, 415 (1945).
7. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159**, 21 (1945).

STUDIES ON THE CYCLOPHORASE SYSTEM

IV. DIRECT DEMONSTRATION OF β OXIDATION

By WILLIAM A. ATCHLEY*

(From the Enzyme Laboratory, Department of Medicine, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, April 28, 1948)

In a previous communication of this series (1), manometric evidence was presented which showed that normal fatty acids with an odd number of carbon atoms and fatty acids of the *iso* series with an even number of carbon atoms were not completely oxidized to carbon dioxide and water. The quantitative data were consistent with the formation of propionic acid in the former case and of isobutyric acid in the latter case as non-oxidizable residues. This interpretation became even more plausible in view of the observation that both propionic acid and isobutyric acid appeared not to be oxidized by the kidney enzyme system. The possibility of identifying these hypothetical residues arising from the oxidation of their parent fatty acids provided the opportunity for a direct demonstration of β oxidation. According to Knoop's classical theory (2) fatty acids are degraded by successive scissions of a 2-carbon unit from the main chain. Accordingly, valeric acid should give rise to propionic acid and isocaproic acid should give rise to isobutyric acid. If, in addition, it could be shown that butyric acid is not an intermediate in the oxidation of valeric acid nor isovaleric acid in the oxidation of isocaproic acid, the evidence for β oxidation would be complete. This communication deals with the demonstration of these points plus the demonstration of propionic acid as an unexpected oxidation product of isobutyric acid.

The resolution and characterization of the fatty acids studied were effected by the method of counter-current distribution as developed by Craig and his coworkers (3–6). The underlying principle of this technique is that compounds with different coefficients of distribution between two immiscible solvents will each exhibit a characteristic behavior when subjected to what is in essence a graded series of extractions in a system of separatory funnels. Not only does this behavior lend itself to the separation of the components of a mixture, but it also permits calculation of the coefficient of distribution for each component. Identification may then be made by comparison of these derived coefficients with those of known

---

* This investigation was supported by grants to D. E. Green from the Rockefeller Foundation, the Williams-Waterman Fund of the Research Corporation, and the American Cancer Society.

compounds, and, conversely, knowing the distribution coefficient of a pure substance, one can accurately predict its behavior in the Craig apparatus for comparison with the observed behavior of an unknown substance.

Results

Oxidation of Valeric Acid—The oxidation of valeric acid with the kidney enzyme system was carried out on a large scale with α-ketoglutaric acid as a cooxidant (*cf.* "Experimental" for details). After the reaction was over, as judged from a small pilot run, the enzyme suspension was deproteinized and the volatile fatty acids separated by distillation from acid solution. The spectrum for the counter-current distribution of the fatty acids is shown in Fig. 1.

The presence of propionic acid is indicated by the curve with its peak in Tube 8, for one can see that its observed values resemble closely those calculated for a pure substance with a distribution coefficient of 0.53, which figure a previous distribution of pure propionic acid had shown to be the distribution coefficient for this substance in this particular solvent system.

The remainder of the valeric acid added originally is represented by the curve with its peak in Tube 22. The observed values were found to fit best a theoretical curve calculated for a substance with a K of 11.3, while the K of a known pure sample of valeric acid had previously been shown to be 9.7 in this system.[1]

A blank run identical with that of the experimental run, save that it was precipitated immediately after the addition of the enzyme, showed the presence of large amounts of valeric acid but no propionic acid.

The propionic acid which has been shown to accumulate from the oxidation of valeric acid must have resulted from the splitting off from the parent compound of a 2-carbon unit, for repeated studies on the oxidation of butyric acid by this enzyme system with manometric techniques have shown that this compound is oxidized to completion (1) and thus could not serve as an intermediate in the oxidation of a substance which gives rise to a non-oxidizable residue.

Accumulation of Acetic Acid—A brief digression is necessary here to explain the peak which is seen at the far left of Fig. 1. This peak was found to be present also in all subsequent experiments and usually corresponded to one which would be given by a substance having a distribution coefficient of about 0.060 to 0.10. Since acetic acid could be shown to have a K of about 0.065 in this solvent pair,[1] this was taken as presumptive evidence that the peak did represent acetic acid. In a later run in which the peak appeared quite free of any contamination by propionic acid, the contents of Tubes 0 to 4 were pooled and the fatty acid separated by distillation. In 0.1 M solution the sodium salt of the fatty acid reacted positively in the lanthanum nitrate test, thus yielding further evidence that the compound responsible for the peak actually was acetic acid. The presence of this

---

[1] At either end of the distribution spectrum it must be noted that the distribution coefficient represents the quotient of a relatively large number divided by a relatively small number, or *vice versa*, and experimental errors are magnified accordingly. In the studies described here, reproducibility of values for K was poor at the ends of the spectrum. It will be seen, however, that those substances which require the most precise identification all lie sufficiently near the center of the spectrum for consistently accurate results.

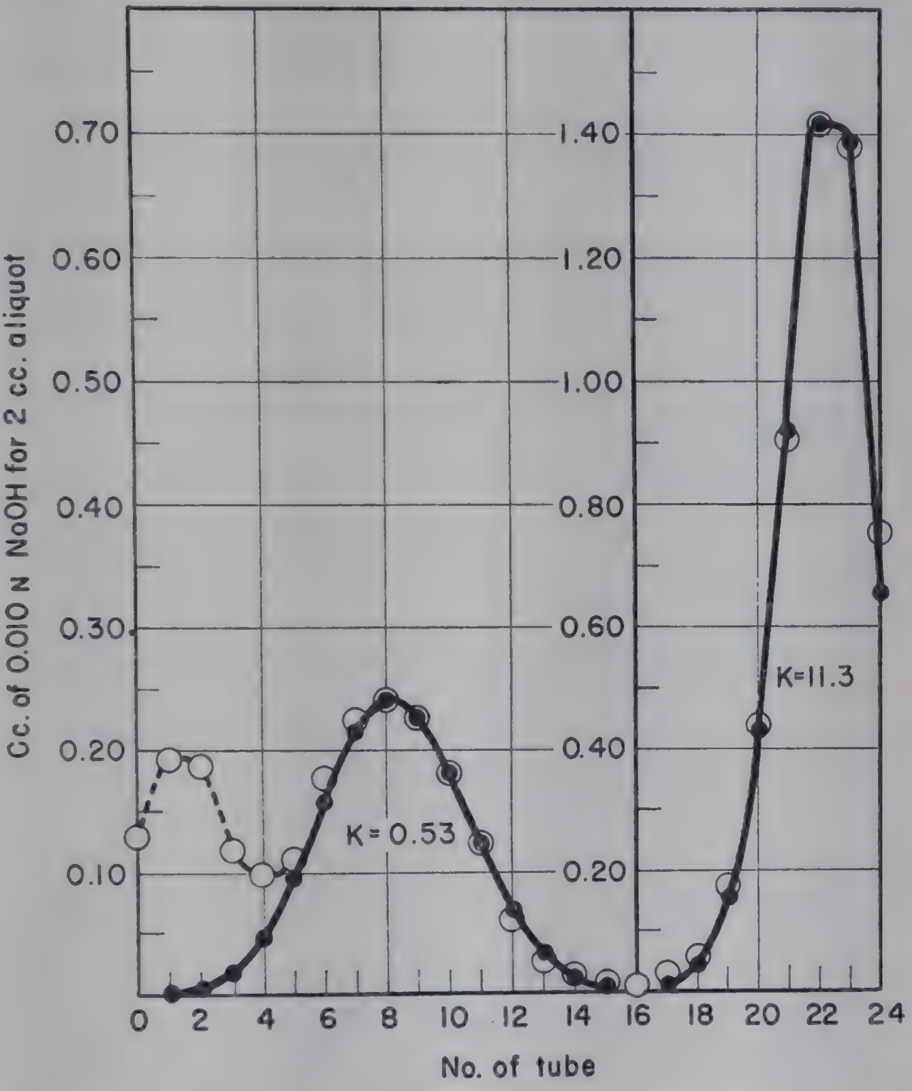


FIG. 1. Oxidation of valeric acid. Solvent system, isopropyl ether and 2.2 M phosphate buffer of pH 5.19. The titration values are for 2 cc. aliquot of the initial extraction. 400 micromoles of valerate were present at the start of the reaction, 214 were present finally. Propionic peak represents about 99 micromoles. ○, observed values; ●, calculated values for K of 0.53 and 11.3, as indicated.

fatty acid was shown by counter-current techniques not to be due to impurities already present in the reagents used in these experiments. It seemed rather to be the resultant of the action of the enzyme on a substrate which either had been added to the reaction mixture or was originally

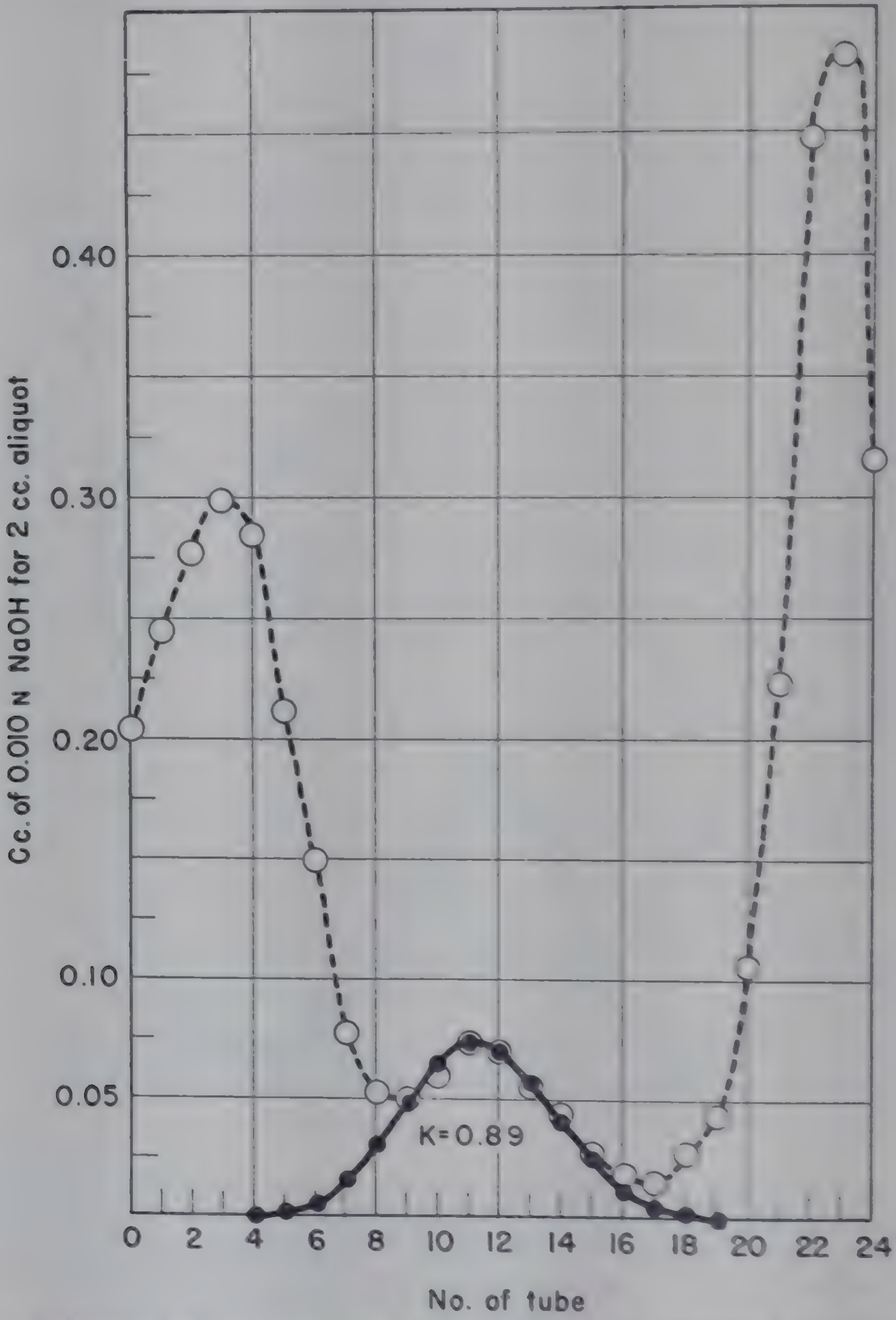


FIG. 2. Oxidation of isocaproic acid. Solvent system, isopropyl ether and 2.2 M phosphate buffer of pH 5.77. The titration values are for 2 cc. aliquot of the initial extraction. 300 micromoles of isocaproic acid were present originally, 67 recovered at the finish. The isobutyric peak represents about 18 micromoles. ○, observed values; ●, calculated values for K of 0.89, as indicated.

present in the enzyme suspension itself. In the studies presented here, no attempt has been made to include acetic acid in any type of balance study.

Oxidation of Isocaproic Acid—When isocaproic acid was oxidized by the kidney enzyme system and the fatty acids present in the reaction mixture at the end of the experiment were analyzed, the results shown in Fig. 2 were obtained. The curve with its peak in Tube 11 can be identified as that of isobutyric acid, for it can be seen that its values fit closely the theoretical values calculated for a pure substance with a distribution coefficient of 0.89, which previous tests outside the machine had shown to be the K value for pure isobutyric acid.

The distribution coefficient of the substance responsible for the curve at the extreme right of the figure can be shown to be about 14. This curve must represent the remainder of the isocaproic acid originally added, the K for this compound having been shown to be 16 in this solvent pair.

The blank for this experiment showed no evidence of the presence of isobutyric acid. It was prepared identically with the experimental run, save that the fatty acid substrate was not added until after the incubation period and just prior to deproteinization.

This accumulation of isobutyric acid as the result of the oxidation of isocaproic acid must have come about by the splitting off of a 2-carbon fragment from the parent fatty acid, since isovaleric acid did not give rise to isobutyric acid under the same experimental conditions.

Oxidation of Isobutyric Acid to Propionic Acid—An examination of Fig. 2, with the analysis of the fatty acids arising from the oxidation of isocaproic acid, shows a curve at the far left which appears to be made up of two separate curves. For this reason, Tubes 0 to 16 were pooled and the fatty acids contained therein were isolated and redistributed between isopropyl ether and phosphate buffer of pH 5.17 instead of 5.77 (Fig. 3). This adjustment of the pH serves to move the peak representing isobutyric acid to the right to Tubes 17 and 18. The values of this new curve fit closely the theoretical curve calculated for a K of 2.6, while that for isobutyric acid in the same solvent pair has been shown to be 2.5.

The original composite curve has been separated by this maneuver into two curves. The one at the left indicates the presence of acetic acid as usual, while that with its peak in Tube 8 represents an unsuspected substance whose observed values lie closest to the theoretical values calculated for a substance with a K of 0.51. This was taken as presumptive evidence that the unsuspected substance was propionic acid, which was shown to have a K of 0.50 in this system.

It was next shown that isobutyric acid on enzymatic oxidation gave rise to this same substance. The distribution curve for one of these experiments is shown in Fig. 4. Here, although a full set of twenty-four transfers has been made, only Tubes 2 to 13 were titrated. The observed values can be seen to lie very close to the theoretical curve calculated for a *K* of 0.52. After distribution the contents of Tubes 5 to 12 were pooled and the fatty acid separated, and, when a 0.05 M solution was tested in the lanthanum nitrate-iodine reaction, the result was strongly positive, thus establishing even more firmly that this compound was propionic acid. Other possible oxidation products of isobutyric acid were examined to determine whether they were similar to the substance uncovered by counter-current distribution. These were methylmalonic acid, β-hydroxyisobutyric acid, α-methylacrylic acid, and α-hydroxyisobutyric acid. The first two of these could

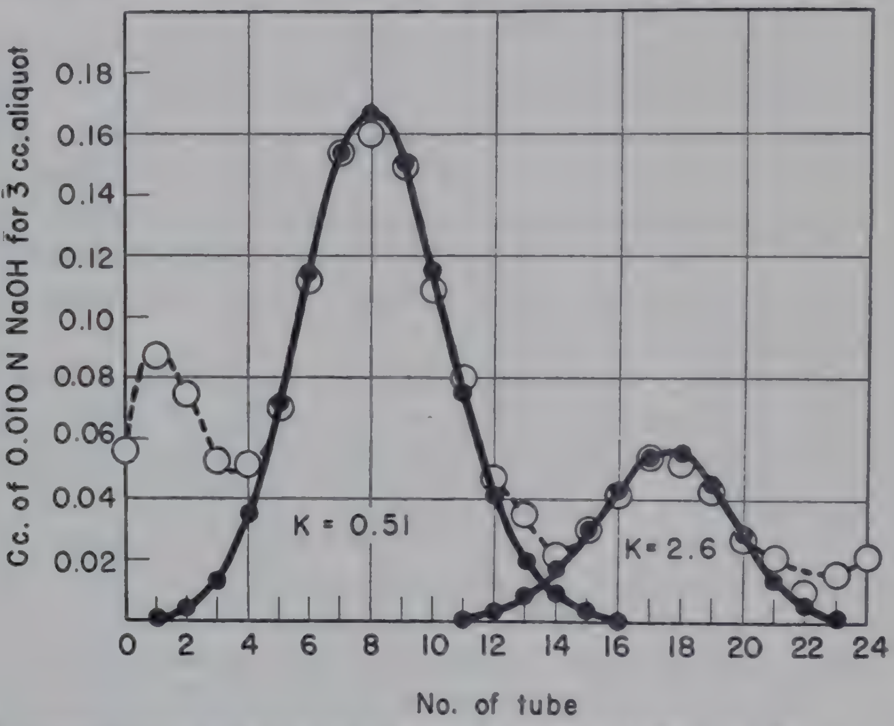
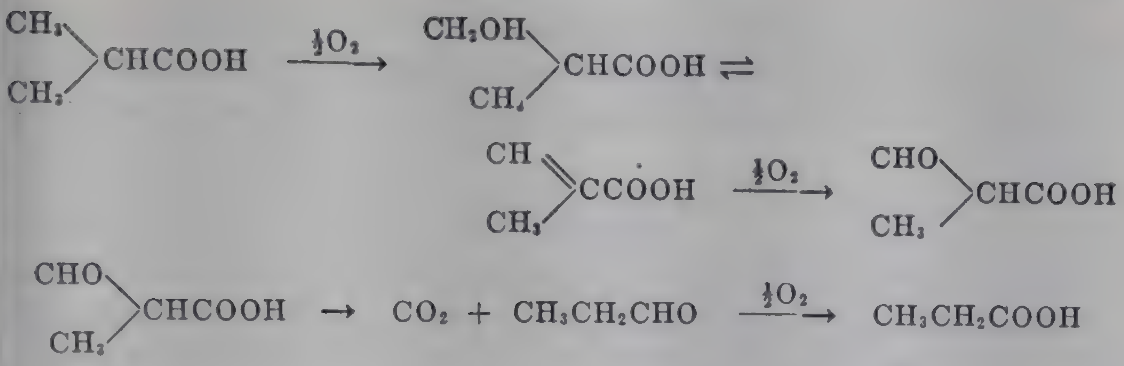


FIG. 3. Redistribution of the acid in Tubes 0 to 16 of Fig. 2. Solvent system, isopropyl ether and 2.2 M phosphate buffer of pH 5.17. The titration values are for 3 cc. aliquot of the initial extraction. ○, observed; ●, calculated for *K* of 0.51 and 2.6, as indicated.

be ruled out because they were not volatile under the conditions of the distillation for the experimental run, while the last two could be ruled out on the basis of their distribution coefficients, which were 1.9 and 0.01 respectively in the solvent pair, the aqueous portion of which was phosphate buffer of pH 5.17.

Mechanism of Isobutyric Acid Oxidation—Assuming that the oxidation of isobutyric acid to propionic acid involves preliminary β oxidation, the accompanying sequence of reactions may be postulated. The liver fatty acid oxidizing system has proved invaluable for demonstrating this mechanism by virtue of its ability to oxidize propionic acid to completion. Thus



$$\begin{matrix}CH_3\\CH_3\end{matrix}\!>\!CHCOOH \xrightarrow{\frac{1}{2}O_2} \begin{matrix}CH_2OH\\CH_3\end{matrix}\!>\!CHCOOH \rightleftharpoons \begin{matrix}CH_2\\CH_3\end{matrix}\!\!>\!CCOOH \xrightarrow{\frac{1}{2}O_2} \begin{matrix}CHO\\CH_3\end{matrix}\!>\!CHCOOH$$

$$\begin{matrix}CHO\\CH_3\end{matrix}\!>\!CHCOOH \rightarrow CO_2 + CH_3CH_2CHO \xrightarrow{\frac{1}{2}O_2} CH_3CH_2COOH$$

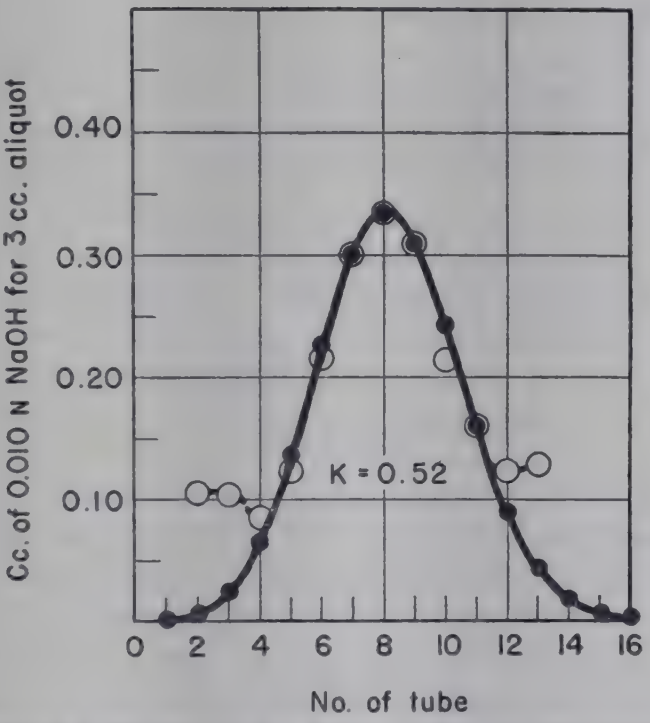


FIG. 4. Oxidation of isobutyric acid. A twenty-four transfer distribution of volatile acids from the final reaction mixture was performed, but only Tubes 2 to 13 were titrated. The solvent system was the same as in Fig. 3. The titration values are for 3 cc. aliquot of the initial extraction. 400 micromoles of isobutyrate were added, and 77 micromoles of propionate recovered. ○, observed; ●, calculated for *K* of 0.52.

any substance which is an intermediate between isobutyric acid and propionic acid should be oxidized to completion in the liver system. Both β-hydroxyisobutyric acid and methylacrylic acid adequately satisfy that requirement. The unresolved point is whether methylmalonic semialdehyde decomposes to propionaldehyde and carbon dioxide or undergoes further oxidation to methylmalonic acid with subsequent formation of propionic acid by decarboxylation. Under the conditions of the experiment, methylmalonic acid appeared completely inactive. Thus the pathway through propionaldehyde is indicated. On structural grounds there is reason to anticipate that methylmalonic semialdehyde would decompose rapidly to propionaldehyde. Consistent with this prediction is the fact that propionaldehyde can undergo extensive oxidation in the liver fatty acid oxidizing system, while only partial oxidation takes place in the kidney system.

EXPERIMENTAL

Enzyme System—In all cases the enzyme used was the triply washed residue of the rabbit kidney homogenate prepared as previously described (6). In its final form this consisted of a thick suspension in 0.9 per cent potassium chloride.

The experimental runs were set up on a scale 20 to 40 times that of the amounts used in a single Warburg cup. A pilot run was always set up to determine the activity of the particular enzyme preparation used and to provide information on the course of the reaction. 1.5 cc. of the enzyme suspension were used in each Warburg cup and this was supplemented by 0.3 cc. of 0.125 M phosphate buffer of pH 7.3, 0.3 cc. of 0.01 M adenosine triphosphate or adenosine monophosphate, and 0.2 cc. of 0.02 M magnesium sulfate. Alkali-soaked filter paper was placed in the center well. In the studies of the oxidation of valeric and isocaproic acids, 10 micromoles of fatty acid substrate were added to each Warburg cup, while 5 micromoles of α-ketoglutaric acid were added as a cooxidant. In the study of isobutyric acid oxidation, 10 micromoles of the cooxidant were added to an equivalent amount of the fatty acid. All substrates were added in the form of their neutral sodium salts. The large scale experiments were carried out in macro manometer cups with oxygen as the gas phase and at 38°.

Preparation of Samples for Distribution—After appropriate incubation periods, the reaction mixtures were removed from the bath and precipitated by the addition of 2.0 cc. of 10 N sulfuric acid and 7.5 cc. of 0.4 M sodium tungstate solution for every 10 cc. of the reaction mixture. In the studies with valeric and isocaproic acids, the fatty acids were separated from the suspension by steam distillation and the distillate was then subjected to another purification distillation after the addition of 2.0 cc. of 10 N sulfuric acid, 50 gm. of magnesium sulfate, and about 0.2 gm. of mercuric oxide. In all the other experiments the original tungstate precipitate was filtered off, the residue washed once, and the combined filtrates then subjected to the purification distillation. As a final step the distillates were neutralized with sodium hydroxide and evaporated to dryness.

Counter-Current Distribution—The machine was the stainless steel type described by Craig (3). In each tube the aqueous phase consisted of 8.0 cc. of 2.2 M phosphate buffer of pH 5.17, 5.19, or 5.88, while the organic phase consisted of 8.0 cc. of isopropyl ether. The sample to be analyzed was taken up in 8.0 cc. of the buffer and added to Tube 0. After twenty-four transfers, the contents of each tube were acidified by the addition of 1.0 cc. of 8 N phosphoric acid and the amount of acid in the ether phase of each estimated by titration of a suitable aliquot with 0.010 N sodium hydroxide in a micro burette.

It is impossible to drive all the fatty acid in each tube into the ether layer for titration by the addition of phosphoric acid as described. Therefore, when it was desired to know the total amount of acid in any tube, it was necessary to extract the buffer layer a second time with 8.0 cc. of ether and again titrate a suitable aliquot. The total amount of acid C can then be calculated from the formula $C = A^2/A-B$ where A is the value for the first titration figure and B is the value for the second (7).

At any point on the curve plotted from the above titration values, the distribution coefficient of the substance responsible for the curve could be determined by the formula

$$K = T_r + 1/T_r \times (r + 1)/(n - r)$$

in which K is the distribution coefficient, r is the number of the tube, T is the titration value for that tube, and n is the number of transfers (in these experiments, always twenty-four).

SUMMARY

Valeric acid and isocaproic acid are oxidized in the kidney fatty acid oxidizing system to propionic acid and isobutyric acid respectively. Part of the isobutyric acid formed is further converted to propionic acid. The end-products were identified by counter-current distribution.

BIBLIOGRAPHY

1. Grafflin, A. L., and Green, D. E., *J. Biol. Chem.*, **176**, 95 (1948).
2. Knoop, F., *Beitr. chem. Physiol. u. Path.*, **6**, 150 (1904).
3. Craig, L. C., *J. Biol. Chem.*, **155**, 519 (1944).
4. Craig, L. C., Golumbic, C., Mighton, H., and Titus, E., *J. Biol. Chem.*, **161**, 321 (1945).
5. Williamson, B., and Craig, L. C., *J. Biol. Chem.*, **168**, 687 (1947).
6. Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, **172**, 389 (1948).
7. Sato, Y., Barry, G. T., and Craig, L. C., *J. Biol. Chem.*, **170**, 501 (1947).

FURTHER STUDIES ON A FAT-SOLUBLE MATERIAL FROM PLASMA HAVING BIOTIN ACTIVITY

BY WILLIAM TRAGER

(*From the Department of Animal and Plant Pathology, The Rockefeller Institute for Medical Research, Princeton*)

(Received for publication, June 18, 1948)

A fat-soluble material from plasma has been found to have biotin-like activity for chicks as well as for lactic acid bacteria (1). Since oleic acid can replace biotin in the nutrition of *Lactobacillus casei* (2, 3) and of a variety of related bacteria (4) as well as in that of a yeast (5), it was of interest to determine whether oleic acid might also have a biotin-like activity for chicks. It is the purpose of this paper to report experiments concerned with this problem and to provide additional data on the biotin activity of the fat-soluble material from plasma, and on its occurrence and partial purification.

EXPERIMENTAL

General Methods—Biotin activity was determined by microbiological assay with *Lactobacillus casei* with the method of Landy and Dicken (6) slightly modified (1). Fat-soluble materials to be assayed were dissolved in warm ethyl alcohol, and distilled water was added to the solution to give a uniform emulsion.

The crude fat-soluble biotin-active material (FSF) was prepared in quantity by the ether extraction of acid-hydrolyzed horse plasma (1). 20 to 30 ml. of a neutral dark brown oil were ordinarily obtained from 10 liters of oxalated plasma. The activity of such preparations ranged from 1.5 to 3 γ of biotin activity per ml., and was usually about 2 γ.

All the chicks used were Rhode Island reds obtained when 1 day old. They were kept in electrically heated brooders until 10 to 14 days old, and then in wire bottom cages in a warm room. The stock diet was a chick starting mash of the following composition: yellow corn-meal 29, ground wheat 29, soy bean meal 17, alfalfa leaf meal 7.5, meat scrap 4.5, cod liver oil 0.7, charcoal 0.7, salt 0.7, calcite 2.1, fine grit 4.4, fine oyster shell 4.4. It contained about 13 γ of biotin per 100 gm. of diet. For the production of biotin deficiency, this mash was mixed with egg white, usually in the proportion of 20 parts of a commercially dried egg albumin in 100 parts of diet. A control casein diet contained in place of egg white washed casein mixed with riboflavin to provide 5 mg. of riboflavin per 100 gm. of casein.

The chicks were kept on the stock diet until they were 5 to 7 days old.

They were then placed on the experimental diets and the treatments, if any, begun. During the period of the experiment each chick received once weekly 3 to 4 drops of haliver oil with viosterol. The treatments consisted usually of the intramuscular injection of biotin, oleic acid, or FSF. The injections were given as 0.2 ml. of liquid in the breast muscle two times per week. The biotin was dissolved at appropriate concentrations in 0.85 per cent sodium chloride solution. The FSF and oleic acid were injected as the warm oils themselves. FSF was absorbed much better than oleic acid, but with both materials abscesses formed in the breast muscle surrounding small pockets of the oil.

At suitable intervals the experimental chicks were weighed and the severity of the dermatitis on the feet and at the corners of the mouth graded by a method similar to that of Ott (7), except that the scale used ranged from 0 to 6. Only occasional chicks, however, showed a dermatitis more severe than grade 4. In order to make this grading as nearly objective as possible, the chicks were taken at random from the various groups by an assistant and presented to the person doing the grading with their number tags covered. The latter individual then gave his judgment of the severity of the dermatitis to the assistant, who noted it beside the appropriate number. Thus the person grading the chicks could not be aware of the nature of the results until all the chicks had been graded. This precaution was desirable, since the experiments with FSF could not be performed in such a way as to give any likelihood of complete prevention of biotin deficiency symptoms. The maximal dosage of FSF which could be administered by injection was far from sufficient, in terms of its microbiological biotin activity, to be expected to provide complete protection from the deficiency. For this reason most of the experiments included two control groups injected with biotin, one with a dosage similar, on an activity basis, to that of the group receiving FSF and one with a dosage adequate (8) to prevent the deficiency entirely. Larger amounts of FSF could be administered by stomach tube, but it has not so far been possible to find any activity of the material when given by this route to chicks on an egg white diet.

Results

Treatment of Chicks on Egg White Diet with FSF and Oleic Acid—The effect previously reported (1) of FSF in mitigating the dermatitis produced in chicks by a diet high in egg white has been repeatedly confirmed. Tables I and II give the results of two experiments of this type, the usual commercial preparation of egg white having been used for one experiment and an acetone-precipitated preparation from fresh egg white (9) for the other. In Table I it will be noted that neither the group receiving FSF nor the groups receiving the lower dosage of biotin gained weight to any greater extent than the untreated group on the egg white diet. Since this was also true in all the other experiments, the weights are not included in Tables II to V. It has been found by others (10) that lower dosages of biotin are required to affect the dermatitis than to permit normal weight gain in chicks.

Preliminary experiments with oleic acid revealed no effect on the dermatitis of chicks on an egg white diet. The results of two experiments comparing FSF preparations with U. S. P. oleic acid and with a soy bean oil distillate (SBO) containing oleic acid[1] are shown in Tables III and IV. Although the oleic acid and the SBO had higher biotin activities for *Lactobacillus casei* than the FSF preparations, neither reduced the extent of the dermatitis, while both FSF preparations did so, one of them (from horse plasma, Table IV) to almost as great an extent as did a comparable dosage of biotin.

TABLE I

Effects of FSF and Biotin on Biotin Deficiency in Chicks Fed Diet High in Egg White

The chicks were started on the special diets and injections when 1 week old.

Diet	Injection (breast muscle)	Weekly dosage, microbiological assay	No. of chicks	Average weight (gm.)					Average degree of dermatitis of feet and mouth		
				7 days	15 days	21 days	28 days	39 days	21 days	28 days	39 days
		γ biotin		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*			
Casein, 20%	None		7	57	98	156	203	281	0	0	0
Egg white, 20%	“		7	58	87	133	163	205	3.4	3.3	4.9
	FSF	0.8	7	58	85	125	150	182	1.7	2.7	3.0
	Biotin	0.8	7	57	96	138	159	205	0.7	0.4	1.6
	“	6.0	6	55	88	140	182	251	0.7	0	0

TABLE II

Effect of Intramuscular Injection of FSF in Chicks on Diet containing 500 Gm. of Dried Acetone-Precipitated Fresh Egg White per 20 Pounds of Feed

Chicks placed on diet when 8 days old, injections begun 3 days later.

Treatment	No. of chicks	Average degree of dermatitis of feet and mouth	
		21 days	28 days
None	8	1.5	2.1
FSF, 0.8 γ biotin activity per wk	9	1.0	1.5

A single somewhat purified preparation of FSF has been tested in chicks and found to be active (Table V). Table V also shows that FSF is active when injected into the posterior peritoneal cavity as well as when injected intramuscularly.

---

[1] The molecular distillate of soy bean oil was very kindly supplied by Dr. P. L. Harris of Distillation Products, Inc.

TABLE III

Intramuscular Injection of U. S. P. Oleic Acid, FSF from Human Plasma Fraction IV-1, and Biotin in Chicks Placed on Egg White Diet When 5 Days Old*

Injections begun at 7 days of age.

Injection	Weekly dosage, by microbiological assay	No. of chicks	Average degree of dermatitis of feet and mouth			
			20 days	30 days	34 days	44 days
	γ biotin					
None	0	7	1.2	3.2	2.9	3.2
Oleic acid	1.2	8	1.3	2.7	2.9	3.6
FSF	0.6	8	1.0	2.8	2.2	2.6
Biotin	0.6	7	1.1	1.7	1.6	1.1
"	6.0	8	0.8	0.7	0.5	0.1

* A large supply of Fraction IV-1 from human plasma was obtained through the generosity of the American Red Cross.

TABLE IV

Comparison of Effects of Intramuscular Injection of Soy Bean Oil Distillate (SBO) containing Oleic Acid, of FSF from Horse Plasma, and of Biotin in Chicks on Egg White Diet

Diet and injections begun at 5 days of age.

Injection	Weekly dosage, by microbiological assay	Average degree of dermatitis of feet and mouth (10 chicks)		
		21 days	28 days	34 days
	γ biotin			
None	0	1.8	2.6	2.9
SBO	1.2	2.0	2.4	2.6
FSF	0.9	1.5	1.7	2.0
Biotin	1.0	1.4	1.3	1.6
"	10.0	0.4	0.1	0.2

Plasma and Tissue Levels of Biotin and Fat-Soluble Biotin-Active Materials—The plasma levels of biotin and FSF in young ducks and chickens and the changes which they undergo during acute infections with avian malaria parasites have been previously described (11). The biotin content of the plasma of normal young ducks and chickens is about 2 to 4 mγ per ml., while the bound FSF content in terms of its biotin activity is about 10 to 15 mγ per ml. No bound biotin and relatively little free FSF have been found in the plasma.

Assays of the plasma of adult birds have shown that, while in males both the biotin and FSF remain much the same as in young birds, in females which are laying eggs both values are increased. Table VI gives a small representative sample of the data which have been obtained and at the

TABLE V

Effects of Injection of FSF from Horse Plasma (Fraction A), of Fraction F Obtained from It by Adsorption on Aluminum Oxide and Elution with Ammoniacal Alcohol, and of Biotin in Chicks on Egg White Diet

Diet and injections begun at 5 days of age.

Injection	Weekly dosage, by microbiological assay	No. of chicks	Average degree of dermatitis of feet and mouth	
			19 days	26 days
	γ biotin			
None	0	5	1.7	2.8
Fraction F*	3.0	7	1.2	2.1
“ A*	1.2	6	1.3	2.2
“ A†	1.2	5	1.4	1.8
Biotin*	1.0	7	0.9	1.5
“ *	10.0	6	0.6	0.4

* Injected to breast muscle.

† Injected to posterior peritoneal cavity.

TABLE VI

Biotin and FSF Content of Plasma of Adult Ducks (5 to 6 Months Old) As Determined in Two Different Ways

Duck		Biotin activity, mγ per ml. plasma					
No.	Sex	Free biotin		Bound biotin	Free FSF	Bound FSF	
		(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)
1	♀	23.3	29.2	0	2.2	34.0	28.1
2	♀	26.0	29.3	−1.3	3.2	38.8	35.5
3	♀	30.0	30.0	−2.5	4.5	31.5	31.5
4	♀	32.0	33.0	−1.8	1.5	27.8	26.8
5	♂	4.7	6.3	−1.7	1.1	17.7	16.1

(*a*) The activity of plasma diluted in water and shaken with ether. (*b*) The activity of plasma hydrolyzed in sulfuric acid minus the activity of the same preparation tested in the presence of 1:500 egg white. (*c*) The activity of plasma hydrolyzed in sulfuric acid and shaken with ether minus the free biotin (*a*). (*d*) The activity of plasma diluted in water minus the free biotin (*a*). (*e*) The activity of plasma hydrolyzed in sulfuric acid minus the activity of plasma diluted in water. (*f*) The activity of plasma hydrolyzed in sulfuric acid and tested in the presence of 1:500 egg white, minus the free FSF.

same time shows the good agreement between the values as determined by removal of FSF with ether and by inactivation of the biotin with egg white. Egg-laying hens showed a high biotin and FSF content of the plasma entirely similar to that of the egg-laying ducks. The increase in biotin may well be related to the high biotin content of the yolk of the egg, which in turn is essential for hatchability (12). In a similar way, avidin secretion by the oviduct accompanies egg-laying activity in hens (13). An increase in the total plasma lipides of egg-laying chickens has been demonstrated (14). Evidently the lipide or lipides with biotin activity share in this increase. It is noteworthy that the free FSF activity remains relatively low in the plasma of the egg-laying birds, most of the increase occurring in the FSF which is bound, presumably as a lipoprotein.

Much of this bound FSF can be precipitated by dilution of the plasma of egg-laying hens or ducks with distilled water in a ratio of 1:20. The protein so precipitated and collected by centrifugation redissolves readily in phosphate buffer of pH 7.4 to give a highly opalescent yellow solution. The bound FSF activity of such solutions, expressed as millimicrograms of biotin per mg. of protein, was 1½ to 3 times as high as that of the original plasma.

Some determinations have been made of the distribution of biotin and fat-soluble biotin-active materials in tissues other than blood. The results for 5 to 6 month-old chickens are presented in Table VII. Since in the growth of *Lactobacillus casei* biotin can be replaced by oleic acid and a variety of related compounds, the microbiological assay of biotin activity due to fat-soluble materials from tissue would determine the sum of the concentration in the tissue of a number of substances. That oleic acid in depot fats did not enter appreciably into the measurements is indicated by the very low FSF activity of the abdominal fat, a tissue which contains about 40 per cent by weight of oleic acid combined as glycerides (15). Whatever the actual substances from tissues may be which have here been measured as FSF, it is remarkable that, except for the liver and kidney which have exceptionally high biotin contents, there is a general parallelism between the biotin content and the relative concentration of the fat-soluble biotin-active materials. A similar situation exists in egg yolk. For example, the yolk of one egg had a total biotin activity of 750 mγ per ml., of which 360 mγ remained after shaking with ether. The figures for biotin proper which are here presented are for the most part somewhat lower than those previously reported, though of the same order of magnitude. Thus Eakin *et al.* (16) found for the organs of a 10 week-old chicken 2600 mγ per gm. in the liver and 2500 mγ in the kidney, but only 65 mγ in the brain. Values for egg yolk have been found ranging from 400 to 600 mγ per ml. (17) with a usual value of about 500 mγ per gm. (12).

Partial Purification of FSF from Horse Plasma—Two different methods have yielded products having as high a specific activity (millimicrograms of microbiological biotin activity per mg. of dry weight) as commercial samples of U. S. P. or C.P. oleic acid. The products so obtained differed

TABLE VII

Biotin and FSF Contents of Representative Tissues from 5 to 6 Month-Old Chickens

Samples of the tissues were ground in saline in a glass grinder, autoclaved in 2.5 N sulfuric acid for 1 hour at 15 pounds, brought to pH 9.6, and diluted with water. The uniform suspensions thus obtained were divided into two portions, one of which was assayed for its total biotin content, while the other was shaken with three to four portions of ether and then assayed. The results of the latter assay gave the biotin content of the preparation, while the difference between the two assays was considered as activity due to FSF. In some cases, indicated by an asterisk, FSF activity was determined directly by the assay of suitable dilutions of the suspension in the presence of egg white.

The results are expressed as millimicrograms of biotin activity per gm. of fresh tissue.

Sex	Chicken No.	Brain	Lymph nodes	Breast muscle	Liver	Abdominal fat	Spleen	Oviduct	Testis	Adrenal glands	Kidney
♀	1	87†	34	13	592	7	45	80			993
		(165)‡	(83)	(43)	(129)*	(10)	(83)	(82)			(122)*
	2	107	57	15	1380	2	72	134			1390
		(203)	(105)	(52)	(160)*	(4)	(98)	(106)			(110)*
	3	97	38	31	780	6	55	179		172	1790
		(195)	(77)	(40)	(131)*	(5)	(116)	(88)		(248)	(74)*
	4	69	37	13	750	4	52	53		112	805
		(160)	(75)	(35)	(102)*	(4)	(82)	(99)		(151)	(60)*
	5	142	49	29	1250	8	68	123			
		(141)	(60)	(48)	(75)	(15)	(102)	(79)			
	6	127	87	36	740	3	77	118		131	1150
		(191)	(106)	(41)		(2)	(89)	(61)		(133)	
♂	7	91	46	19	850	15	42			105	895
		(168)	(65)	(35)	(62)*	(8)	(103)			(140)	(56)*
	8	87	187	10	605		38			150	1675
		(174)	(58)	(43)	(64)*		(99)			(136)*	(72)*
	9	126	80	27	1220	13	64			170	2140
		(189)	(101)	(39)		(17)	(110)			(142)	
	10	82	44	18	1210	5	42			211	1625
		(228)	(76)	(34)	(60)	(4)	(88)				(0)
	11		197	24	1040		91		66	86	1123
			(100)	(42)	(180)		(71)		(114)	(151)	(162)
Averages...		101	78	21	947	7	59	114		142	1369
		(181)	(83)	(41)	(107)	(7)	(95)	(86)		(157)	(82)

† The upper figures represent biotin throughout.

‡ The lower figures represent FSF throughout.

obviously from oleic acid in being at ordinary room temperature (20–25°) whitish or pale yellow waxy solids.

One method was a crude counter-current distribution employing nine separatory funnels (18). Table VIII illustrates the results of such an experiment. The starting material in this case was prepared by fractionation in alcohol. 75 ml. of the oil from hydrolyzed horse plasma were suspended in 200 ml. of hot alcohol. The insoluble portion was extracted with two more portions of hot alcohol. The combined alcohol solutions were refrigerated overnight and filtered in the cold until clear. The portion soluble in cold alcohol, which contained virtually all of the activity, was concentrated *in vacuo* to remove the alcohol. A dark brown oil was obtained which solidified in the cold. 1 ml. of the oil was dissolved in cyclohexane (previously shaken with 90 per cent ethyl alcohol) to a volume of 10 ml. This solution was then used for an eight plate distribution between cyclohexane and 90 per cent ethyl alcohol, each of which had been previously thoroughly shaken with the other solvent. A theoretical curve based on the distribution coefficient (about 1.1) of the biotin activity between cyclohexane and 90 per cent alcohol would have placed the maximal amount of activity in Funnel 4. Actually, it was distributed between Funnels 2 to 4. However, the highest specific activity occurred in Funnel 5. In another similar experiment the maximal amount of activity was distributed between Funnels 3 to 5 and the specific activity of the material from Funnel 4 was 10 mγ per mg. A sample of U. S. P. oleic acid dissolved in alcohol, which was assayed at the same time with this fraction and for which the dry weight

TABLE VIII

Counter-Current Distribution of FSF Activity in Nine Separatory Funnels between Cyclohexane and 90 per Cent Alcohol

Funnel No.	Total activity for *Lactobacillus casei* when tested at concentrations of 1:2000 and less	Specific activity
	mγ	*mγ per mg. dry weight*
0	0	
1	420	
2	900	4.1
3	880	4.0
4	820	5.1
5	540	6.8
6	360	
7	60	
8	0	
Total recovered out of 3750 mγ...	3980	

was determined in the same manner, gave a specific activity of 10.9 mγ per mg. However, in other assays the specific activity of oleic acid would be as low as 7 mγ per mg. It is important to note here that some fluctuation in the assay results is introduced as a result of the toxicity of higher concentrations of the fat-soluble materials. The toxic effect was relatively small with most of the preparations of the original oil from hydrolyzed horse plasma, so that at suitable concentrations nearly maximal growth was obtained. Moreover, with these crude FSF preparations the dose-response curve closely approached that with biotin itself (1). Such is not the case with oleic acid (2) or with the more highly purified preparations of FSF. Higher concentrations of these substances all exhibit a growth-inhibiting effect on *Lactobacillus casei*, before maximal growth has been attained.

TABLE IX

Chromatographic Adsorption of FSF from Alcoholic Solution onto Aluminum Oxide

Solvent passing through column	Fraction No. (each collected from bottom of column for 10 min. period)	Total biotin activity for *L. casei*	Specific activity
		mγ	*mγ per mg. dry weight*
95% ethyl alcohol	1, 2	0	
	3	10	
	4–8	0	
5% ammonia in 66% ethyl alcohol	9–12	0	
	13	15	
	14	45	9.0
	15	70	10.8
	16	60	9.2
Starting material		1920	3.2

The second method of partial purification depended on chromatographic adsorption on aluminum oxide and elution with ammoniacal alcohol. The activity could be adsorbed either from a cyclohexane solution or from an alcoholic solution containing only that portion of the original oil which was soluble in cold alcohol. When such a solution was allowed to percolate through a 10 × 2 cm. column of aluminum oxide prepared in the corresponding solvent and was followed by more of the same solvent, an orange-yellow zone soon formed and migrated down the column at a fairly rapid rate. All the percolates appeared colorless, except those which included the zone and which were bright yellow. In repeated trials, the fraction containing the colored material has had a low but significant activity, whereas the fractions preceding and following it have had little or no activity. Most of the activity of the original solution, however, remained adsorbed as long as cyclohexane, 95 per cent alcohol, or alcohol acidified with HCl was passed through the column. Elution began soon after the addition of ammoniacal alcohol (5 per cent ammonia in 66 per cent ethyl alcohol) and continued slowly over a considerable period. Tables IX and X give the results of two experiments of this type. In the experiment illustrated in Table IX, the yellow material was collected as a separate fraction (No. 3). It is obvious that in this experiment elution was still occurring at an appreciable rate when the collection of fractions was discontinued. The high specific activity of Fractions 14 to 16 is worthy of note. In the larger scale experiment illustrated in Table X the yellow material was not collected separately but it probably was responsible for most of the 460 mγ of activity present in the combined percolate from the sample and the following 100 ml. of alcohol.

TABLE X

Chromatographic Adsorption of FSF from Alcoholic Solution onto Aluminum Oxide

Material	Total activity	Specific activity
	mγ	*mγ per mg. dry weight*
Starting material. Cold alcohol-soluble portion of oil from hydrolyzed horse plasma in 95% alcohol	20,000	
Combined percolates from sample and 100 ml. 95% alcohol	460	
1st hr.'s percolate after start of 5% NH_3 in 66% alcohol	270	
Next 20 min. percolate	1,000	3.2
" 20 " "	1,000	5.4
" 20 " "	800	
" 20 " "	800	6.7
" 20 " "	800	6.7
Total recovered in percolates..............	5,130	

A larger quantity of a partially purified material was prepared by gross adsorption and elution. 95 ml. of the oil from horse plasma were dissolved in cyclohexane to 400 ml. 150 gm. of aluminum oxide were added and the mixture was stirred for half an hour. It was then filtered. To the filtrate were added an additional 100 gm. of aluminum oxide, and the mixture was again stirred for half an hour. It was filtered through the same paper which had been used previously, and the combined residues were washed with 300 ml. of cyclohexane. The combined filtrates were concentrated *in vacuo* at 45–50° to give 50 ml. of a brown oil with about one-fourth the activity of the starting material. The aluminum oxide residue was suspended in 560 ml. of 5 per cent ammonia in 66 per cent ethyl alcohol and shaken mechanically half an hour. The aluminum oxide was allowed to settle and the supernatant was poured off through a filter paper. The aluminum oxide was then resuspended in an additional 280 ml. of ammoniacal alcohol and was again shaken mechanically for half an hour. The mixture was filtered. The combined clear amber filtrates were brought to a pH of 7 with 6 N HCl to give a cloudy liquid with droplets of brown oil. This was shaken in 100 ml. portions with 100 ml. of cyclohexane in a separatory funnel. The same cyclohexane was used for three portions of the neutralized eluate. All of the aqueous-alcoholic lower layers were pooled. On concentration of these *in vacuo* at 50° droplets of brown oil appeared when the volume was about 200 ml. This material was taken up in ether. On removal of the ether there were obtained 4 ml. of dark brown oil with an activity of 6.5 γ per ml. The combined cyclohexane extracts were concentrated *in vacuo* at room temperature to a small volume and left overnight. A light brown waxy material was obtained which, when warmed, became an easily flowing light brown liquid. 12 ml. of this material were obtained, with a biotin activity for *Lactobacillus casei* of 7.6 γ per ml. This was the fraction (F) which was found to have biotin-like activity for chicks (see Table V).

DISCUSSION

The experiments reported in the present paper support the conclusion that hydrolyzed plasma yields a substance, readily extractable with ether, which, when injected intramuscularly, has biotin-like activity in reducing the extent of the dermatitis produced in young chicks by a diet high in egg white. Usually the effect was not as great as that obtained by the injection of a comparable dosage of biotin in terms of microbiological biotin activity. Several explanations for this may be suggested. In the first place, while there was never any leakage of the injected saline-biotin solutions on withdrawal of the needle, some leakage of the oils with FSF activity frequently occurred. While the saline was rapidly and completely absorbed, this was not true of the oil, which moreover had some toxic properties. Finally, it is possible that the oil obtained from hydrolyzed horse plasma contains more than one substance with biotin activity for *Lactobacillus casei* but only one with biotin activity for chickens. This possibility is supported by the high biotin activity for *Lactobacillus casei* of oleic acid, a substance with which it has not been possible to demonstrate any biotin activity in chickens.

In the studies directed toward purification of the active material in FSF the activity was followed only by microbiological assay, since amounts sufficient for assay in chicks could not readily be obtained. It was hoped that methods in which the material was subjected to relatively mild treatments would yield final products retaining their activity for chickens. This hope was realized with the partly purified fraction (F) obtained by adsorption and elution. More drastic procedures such as saponification were avoided, although in the first work concerning the fat-soluble biotin-active substance from hydrolyzed plasma it was found that the activity for *Lactobacillus casei* remained in the unsaponified fraction (1). Hofmann and Axelrod (19), in their note confirming the finding of a neutral oil from plasma with biotin activity, reported that the activity went into the saponifiable fraction. On the basis of personal communication, it would appear that this discrepancy is the result of a longer period of heating with alkali employed by the latter workers than by the former, who heated for 45 minutes. With several hours heating, the activity of FSF for *Lactobacillus casei* goes into the saponifiable fraction. Such saponified material has not been tested for its activity in chicks.

A number of authors have suggested ways in which oleic acid and related compounds might function in bacterial metabolism. Dubos (20) has concluded that in the growth of his *Micrococcus* C they probably act as catalysts, whereas in the growth of tubercle bacilli they enter into the synthesis of cell protoplasm. Guirard *et al.* (21) have noted their rôle as substitutes for acetate in the nutrition of some of the lactic acid bacteria. Still other lactic acid bacteria require oleic acid in addition to both biotin and acetate (4, 22, 23). Williams and Fieger (3) have postulated that both biotin and oleic acid function as cell permeability factors. Perhaps somewhat more likely is the hypothesis (4) that biotin is essential for the synthesis of oleic acid as it is for the synthesis of aspartic acid (24, 25).

The latter hypothesis could readily be applied to multicellular organisms, perhaps with the further addition that biotin enters into more than one stage of the synthesis of fatty compounds. It was early noted by Boas (26) that rats with severe biotin deficiency had almost no stores of body fat. Fatty infiltration of the liver and increase in body fat of rats have been observed after biotin administration (27), and more recently it has been found that rats with incipient biotin deficiency, unlike normal rats, did not store excess lipide in the liver when fed a diet high in cholesterol (28). The activity of the neutral oil from hydrolyzed horse plasma for chickens as compared to the inactivity of oleic acid for these animals suggests that perhaps in vertebrates the fatty acid must be supplied already combined in some manner in order to eliminate partially the need for biotin. It is also possible that the neutral oil is effective merely because it provides the fatty acid in less toxic form (4). In this connection it is interesting to note that for a quite different metazoan organism, the larva of the yellow fever mosquito, it has been found that, while both oleic acid and FSF can replace biotin, the former material is active over a narrower range than the latter, presumably because of its greater toxicity.[2]

SUMMARY

The intramuscular injection of a fat-soluble material from hydrolyzed plasma into chicks fed a diet high in egg white reduced the severity of their dermatitis almost as much as did the injection of biotin in a comparable dosage in terms of microbiological biotin activity. The similar injection of oleic acid did not have such an effect, nor could the effect be produced by the oral administration of the material from plasma.

The concentration of the fat-soluble biotin-active material, as measured by microbiological assay in the plasma of ducks and chickens, shares in the general increase in lipides which occurs with the onset of egg-laying activity. The distribution of this material in the tissues of birds roughly parallels the distribution of biotin, except in the liver and kidney, which contain relatively very large amounts of biotin. Much of the bound fat-soluble biotin-active material in the plasma of egg-laying hens may be concentrated in a protein fraction precipitated by dilution of the plasma with water.

Partial purification of the active material from hydrolyzed horse plasma has been effected by counter-current distribution in separatory funnels and by chromatographic adsorption. Fractions have been obtained which have as high a specific activity for *Lactobacillus casei* as oleic acid but which differ from oleic acid in physical properties. A somewhat similar fraction prepared by gross adsorption and elution has been shown to have the biotin-like activity when injected into chicks on an egg white diet.

BIBLIOGRAPHY

1. Trager, W., *Proc. Soc. Exp. Biol. and Med.*, **64,** 129 (1947).
2. Williams, V. R., and Fieger, E. A., *J. Biol. Chem.*, **166,** 335 (1946).
3. Williams, V. R., and Fieger, E. A., *J. Biol. Chem.*, **170,** 399 (1947).
4. Williams, W. L., Broquist, H. P., and Snell, E. E., *J. Biol. Chem.*, **170,** 619 (1947).
5. Axelrod, A. E., Hofmann, K., and Daubert, B. F., *J. Biol. Chem.*, **169,** 761 (1947).
6. Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, **27,** 1086 (1942).
7. Ott, W. H., *J. Biol. Chem.*, **157,** 131 (1945).
8. Hegsted, D. M., Mills, R. C., Briggs, G. M., Elvehjem, C. A., and Hart, E. B., *J. Nutr.*, **23,** 175 (1942).
9. Eakin, R. E., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **140,** 535 (1941).
10. Richardson, L. R., Hogan, A. G., and Miller, O. N., *Univ. Missouri Agr. Exp. Sta., Res. Bull. 343* (1942).
11. Trager, W., *J. Exp. Med.*, **85,** 663 (1947).
12. Couch, J. R., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *J. Nutr.*, **35,** 57 (1948).

---

[2] Trager, W., unpublished.

13. Hertz, R., *Physiol. Rev.*, **26,** 479 (1946).
14. Lorenz, F. W., Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, **122,** 619 (1937–38).
15. Hilditch, T. P., The chemical constitution of natural fats, London (1940).
16. Eakin, R. E., McKinley, W. A., and Williams, R. J., *Science*, **92,** 224 (1940).
17. György, P., and Rose, C. S., *Proc. Soc. Exp. Biol. and Med.*, **49,** 294 (1942).
18. Craig, L. C., Golumbic, C., Mighton, H., and Titus, E., *J. Biol. Chem.*, **161,** 321 (1945).
19. Hofmann, K., and Axelrod, A. E., *Arch. Biochem.*, **14,** 482 (1947).
20. Dubos, R. J., *J. Exp. Med.*, **85,** 9 (1947).
21. Guirard, B. M., Snell, E. E., and Williams, R. J., *Arch. Biochem.*, **9,** 361 (1946).
22. Hutchings, B. L., and Boggiano, E., *J. Biol. Chem.*, **169,** 229 (1947).
23. Whitehill, A. R., Oleson, J. J., and SubbaRow, Y., *Arch. Biochem.*, **15,** 31 (1947).
24. Koser, S. A., Wright, M. H., and Dorfman, A., *Proc. Soc. Exp. Biol. and Med.*, **51,** 204 (1942).
25. Stokes, J. L., Larsen, A., and Gunness, M., *J. Bacteriol.*, **54,** 219 (1947).
26. Boas, M. A., *Biochem. J.*, **21,** 712 (1927).
27. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **141,** 619 (1941).
28. Okey, R., *J. Biol. Chem.*, **165,** 383 (1946).

ELECTROMETRIC AND COLORIMETRIC DETERMINATION OF CARBONIC ANHYDRASE

By KARL M. WILBUR AND NORMAN G. ANDERSON

(*From the Department of Zoology, Duke University, Durham, North Carolina*)

(Received for publication, June 4, 1948)

The activity of carbonic anhydrase has been estimated by three procedures: manometric (1–3), colorimetric (2, 4–8), and electrometric (9). For investigations of the enzyme concentration of tissues the colorimetric method has often been used in preference to the manometric technique because of its relative simplicity. A disadvantage of the colorimetric method has been the inhibitory effects of the indicator (10) and of the carbonate-bicarbonate buffer, an effect also true of phosphate buffer in the manometric method, though to a lesser degree (8). Roughton and Booth (8) have found that veronal buffer is without noticeable effect on carbonic anhydrase and have suggested a colorimetric method which employs this buffer with brom-thymol blue. This method permits the measurement of enzyme activity in terms of moles of CO_2 hydrated, allowance being made for inhibition by the indicator. However, the influence of the indicator on the enzyme activity in this system has not been studied.

This paper describes a new electrometric method which is here employed for evaluation of the inhibitory effect of indicators on carbonic anhydrase preparations from various sources. A modification of the veronal colorimetric method suitable for routine determinations of carbonic anhydrase in tissues is also presented together with data on its reliability.

Electrometric Method

In the electrometric method suitable amounts of veronal buffer and saturated CO_2 solution are mixed at 0° to give a displacement of the hydrogen ion concentration from approximately pH 8 to 6.3 in a conveniently measurable period of 100 to 120 seconds for the uncatalyzed reaction. The apparatus employed (Fig. 1) utilizes two automatic measuring syringes (Becton, Dickinson improved Cornwall syringe No. 1250) to force buffer and CO_2 solutions simultaneously into a reaction vessel containing a glass electrode and the enzyme or experimental solutions or both. The pH changes may be followed (Fig. 2) or the time necessary for the solution to reach the end-point (pH 6.3) determined. After the reaction is completed, the reaction vessel is flushed and drained through a distilled water-evacuation system.

Veronal Buffer—4.536 gm. of sodium barbiturate were dissolved in 950 ml. of double distilled water, and barbituric acid was added to bring the pH to 8.15. The amount required varied slightly with the source. The solution was then made up to 1 liter in a glass-stoppered flask. Precautions were taken to prevent carbon dioxide absorption.

Saturated CO_2 Solution—CO_2 from a Dewar flask containing solid CO_2 was bubbled through a gas bubbler into double distilled water at 0° for at least 1 hour before use.

Indicators—To study the effect of indicators on the catalyzed and uncatalyzed reactions, powdered brom-thymol blue (Harleco) and powdered phenol red (Merck) were made up in double distilled water and adjusted to pH 6.3 with NaOH.

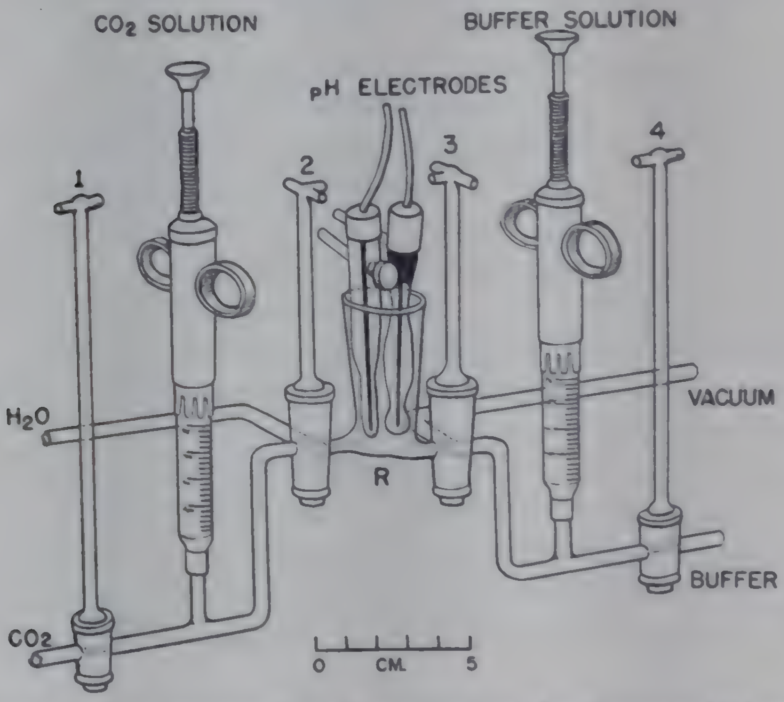


FIG. 1. Apparatus for electrometric determination of carbonic anhydrase activity

Apparatus—Those portions of the apparatus shown in Fig. 1 are mounted on a metal support and placed in a glass container, 12 × 7½ × 8¾ inches. Connected with the apparatus are (*a*) a 250 ml. Mariotte bottle of CO_2-saturated distilled water with a saturating device and a long tube to remove excess CO_2 from the vicinity of the apparatus, (*b*) a 250 ml. bottle of veronal buffer with an air vent protected by an NaOH solution, (*c*) a glass cooling coil connected with an elevated distilled water supply, and (*d*) a vacuum line. The total assembly was packed in a water-ice mixture in the glass container and placed in a wooden box with glass wool insulation between the two containers. It was found convenient to carry out the measurements in a refrigerated room at 4°. All experimental solutions were kept at 0°.

Standard Beckman electrodes were mounted on a rack and pinion over the reaction vessel and connected through a shielded cable to a Leeds and Northrup pH meter. The pH meter and electrodes were standardized in position at 0° with phosphate buffers. All solutions used were made with double distilled water.

Operation—To place the system in operation, the plunger of the buffer syringe is removed and positive pressure induced through the CO_2 absorption system of the buffer bottle to fill the syringe through Stop-cock 4. (The construction of the stop-cock extensions indicates the orientation of the stop-cock.) The syringe is then reassembled and the contents forced into the reaction vessel (*R*) through Stop-cock 3 after Stop-cock 4 is closed. With the syringe plunger still down, Stop-cock 3 is turned to neutral position and Stop-cock 4 is opened. The slow release of the plunger allows the syringe to fill with fresh buffer. Stop-cock 4 is then closed. A similar procedure is used to fill the other side of the system with saturated CO_2 solution. With care all bubbles can be eliminated from the system.

The reaction vessel and electrodes are flushed in the following manner. After both sides of the system are filled, Stop-cocks 1 and 4 are closed and Stop-cocks 2 and 3 turned to the positions shown. Cold distilled water is allowed to flow into the reaction vessel through Stop-cock 2 until it is full. The stop-cock in the vacuum line (not shown) is then opened and the water withdrawn through Stop-cock 3 which is low enough for complete drainage. This process is repeated until the vessel and electrodes are clean (see below).

In making a determination all stop-cocks are turned to neutral position. 1.0 to 2.0 ml. of experimental solution or distilled water are carefully pipetted to the bottom of the reaction vessel. Both openings into the reaction vessel are covered by this quantity of solution, so that both the buffer and CO_2 solutions flow in under liquid. After temperature equilibrium is reached in this solution Stop-cocks 2 and 3 are turned to connect the reaction vessel with both syringes. Both plungers are depressed simultaneously, giving rapid mixing. Stop-cocks 2 and 3 are turned at once to neutral position and Stop-cocks 1 and 4 opened. Both plungers are then slowly released, refilling the syringes, and Stop-cocks 1 and 4 are then closed.

EXPERIMENTAL

Methods—Carbonic anhydrase preparations were made from human blood, gastric mucosa of *Rana pipiens*, and from whole rat brain. For blood carbonic anhydrase, hemolyzed erythrocytes and the modified crude chloroform extract of Roughton and Booth (11) were used. Neither preparation showed appreciable change in activity when kept in a refrigerator several weeks in dilute solution. Mucosal and brain extracts were made by grinding fresh tissue thoroughly and diluting with about 15 volumes of distilled water. The suspension was then centrifuged 8 minutes at 850 × *g* and the supernatant removed and diluted to give a reaction time of 45 to 70 seconds.

0.2 ml. of enzyme solution was added to 1.0 ml. of experimental solution or distilled water in the reaction vessel 30 seconds prior to the addition of 2.0 ml. each of CO_2 solution and veronal buffer. Contact of enzyme and indicator was limited to this period plus the time of reaction.

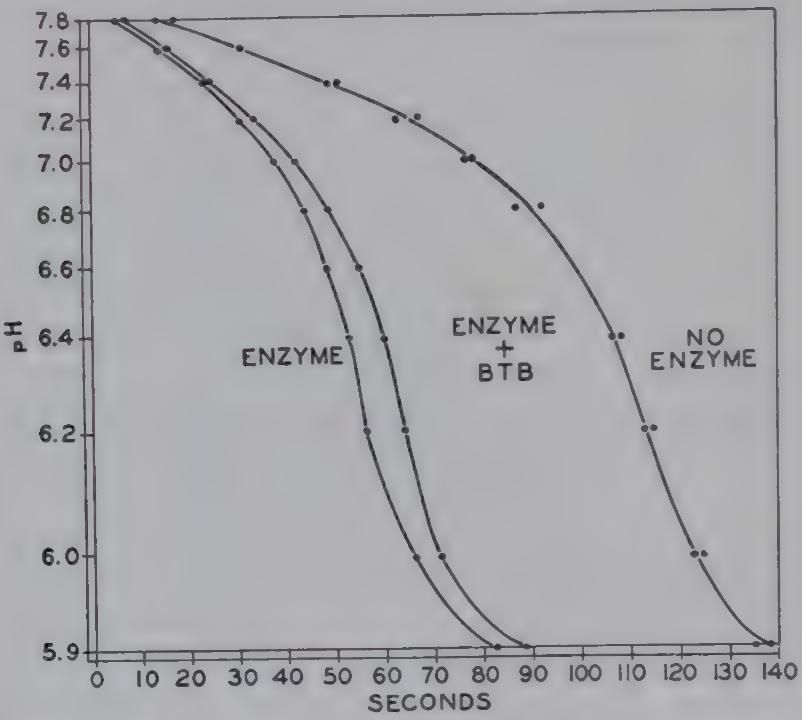


FIG. 2. Hydration of carbon dioxide in veronal buffer. Displacement of curve of enzyme plus brom-thymol blue indicates slight inhibition.

Results—The curves of a catalyzed and an uncatalyzed reaction are shown in Fig. 2; duplicate determinations were performed for the uncatalyzed reaction. As illustrated by the curves, the rate of change resulting from the low buffering power at pH 6.3 makes this a suitable end-point for both the electrometric method and the colorimetric method of Roughton and Booth (8) with brom-thymol blue.

The possibility of enzyme adsorption on the walls of the reaction vessel and the glass electrodes was examined by running a very potent crude blood preparation and then measuring the rate of the uncatalyzed reaction which followed after three and again after six flushings of the reaction vessel. Six flushings appear adequate.

Brom-thymol blue has an inhibitory action on carbonic anhydrase, the extent depending upon the indicator concentration and the particular enzyme preparation (Table I). With 1.92 mg. per cent of indicator, a concentration suitable for colorimetric determinations, the inhibition on rat brain and frog gastric mucosa preparations was about 11 per cent and 8 per cent respectively, with a negligible effect on blood preparations. In a similar series with phenol red (Table II) in a concentration of 2.88 mg. per cent, as ordinarily used in the Brinkman technique, the indicator was without significant effect except in the case of hemolyzed blood in which there was an 8 per cent inhibition.

TABLE I

Inhibition of Carbonic Anhydrase by Brom-thymol Blue

All measurements were made with veronal buffer and electrometric equipment at 0°. The pH of the added indicator solution was adjusted at 6.3. The figures for the indicator represent final concentrations. Runs with enzyme and enzyme plus indicator were alternated.

Enzyme preparation	Indicator	Enzyme + water	Enzyme + indicator	Per cent inhibition	Probability
	mg. per cent	*sec.*	*sec.*		
Rat brain	1.92	53.8	59.5	11	$0.001 < P < 0.01$
	3.84	48.4	62.9	30	$P < 0.001$
Frog gastric mucosa	1.92	52.3	56.5	8	$0.02 < P < 0.05$
	3.84	51.1	62.6	23	$0.001 < P < 0.01$
Human blood, hemolyzed	[illegible].92	51.2	52.0		$0.5 < P < 0.6$
	3.84	54.3	60.3	11	$0.001 < P < 0.01$
Human blood, chloroform extract	1.92	51.7	53.1	3	$0.4 < P < 0.5$
	3.84	53.8	56.0	4	$0.1 < P < 0.2$
No enzyme	3.84	113.2	113.7		$P < 0.001$

TABLE II

Inhibition of Carbonic Anhydrase by Phenol Red

All measurements were made with veronal buffer and electrometric equipment at 0°. The added indicator solution was adjusted to pH 6.3. Final concentration of phenol red was 2.88 mg. per cent. Runs with enzyme and enzyme plus indicator were alternated.

Enzyme source	Enzyme + water	Enzyme + indicator	Per cent inhibition	Probability
	sec.	*sec.*		
Rat brain	56.7	57.9		$0.7 < P < 0.8$
Human blood, hemolyzed	56.5	61.2	8	$0.01 < P < 0.02$
" " chloroform extract	69.1	71.3	3	$0.05 < P < 0.1$
No enzyme	115.2	114.1		$0.7 < P < 0.8$

Colorimetric Method

Apparatus and Procedure—The colorimetric method is an adaptation of that described by Roughton and Booth (8) and utilizes a Cornwall automatic syringe to introduce cold saturated CO_2 solution into a veronal buffer containing brom-thymol blue in a test-tube. The time required for the pH to drop from approximately pH 8 to 6.3 is measured.

The CO_2 saturation system was similar to that used for the electrometric method. In making a determination, 2.0 ml. of saturated CO_2 solution are drawn into a cold syringe, and a small test-tube is placed over a 3 inch No. 20 gage needle and held by a small rubber stopper on the shaft of the needle. The syringe is then immersed in a water-ice mixture until needed. 2.0 ml. of the cold veronal buffer used in the electrometric method containing 5 mg. per cent of brom-thymol blue are then pipetted into a Kimball test-tube, 6 × $\frac{3}{4}$ inches, with 1.0 ml. of the experimental solution or distilled water, stoppered, and placed in an ice-water mixture. After allowing time for temperature equilibration in the test-tube and the syringe, the syringe is quickly removed from the ice mixture, grasped with a cloth to remove excess water, and the small tube protecting the needle is pulled off. The CO_2 solution is rapidly ejected. With the size of test-tube indicated for the reaction vessel the syringe will come to rest with the tip of the needle immersed near the middle of the buffer solution and centered by the rubber stopper on the needle. After removing the syringe from the test-tube the needle is washed to remove any enzyme before the plunger is released. The needle may be coated with paraffin, though this was not done in the present study. The end-point is determined by matching with a comparator tube containing brom-thymol blue in veronal buffer at pH 6.3. Fogging of the ice bath can be prevented by Anti-Fog, No. 111, supplied by the American Optical Company.

Results—Eleven consecutive determinations of the uncatalyzed reaction gave an average of 103.5 seconds with a probable error of 1.0 second for a single determination. Two series of five runs each with enzyme gave averages of 86.4 and 80.4 seconds, with a probable error of 1.2 and 2.3 seconds for a single determination. Measurements were made with ice-packed equipment in a room at normal temperature.

DISCUSSION

The electrometric method with its provision for the automatic measurement of solutions and washing of the reaction chamber permits the measurement of the carbonic anhydrase activity of a considerable number of samples in a relatively short period of time. While the method has the disadvantage that the enzyme is subjected to a range of hydrogen ion concentrations during the course of a single measurement, this range may be limited to any desired portion of the curve.

The electrometric apparatus may be used without the electrodes in colorimetric determinations by adding indicator to the stock buffer solution, though its chief application is with turbid or colored solutions in which brom-thymol blue cannot be used or in which details of the course of the reaction are required. It has been used here to test the effects of indicators on enzymes under conditions which duplicate the colorimetric technique in pH range, indicator concentration, and volume of solutions.

The effects of brom-thymol blue and phenol red on different carbonic anhydrase extracts call attention to certain considerations in the use of colorimetric methods. Since the extent of inhibition varies with the tissue extract, this value will have to be determined for the individual tissue before corrections can be made in calculations of enzyme activity by the procedure of Roughton and Booth (8). In any case, it is apparent that in the colorimetric method the indicator concentration should be kept as low as is practicable. The effects here described relate to the experimental situation in which indicator is mixed with enzyme immediately prior to the addition of the substrate, and may or may not obtain with prolonged contact. We are unable to say whether the marked inhibition found by Kiese and Hastings (10) may involve this factor or whether it is due to differences in extract and experimental method.

SUMMARY

1. An electrometric technique for the measurement of carbonic anhydrase activity is described. In the apparatus employed saturated carbon dioxide and veronal buffer are mixed with automatic measuring syringes and the pH change measured with the glass electrode.

2. The electrometric method was employed to study the inhibitory effect of brom-thymol blue and phenol red on the carbonic anhydrase activity of rat brain, frog gastric mucosa, and human erythrocyte preparations; the extent of inhibition was found to vary with the enzyme source and indicator concentration. An indicator concentration suitable for use in a colorimetric technique gave an inhibition of 0 to 11 per cent.

3. A modification of the Roughton and Booth veronal-brom-thymol blue colorimetric system is described and data on its reliability presented.

BIBLIOGRAPHY

1. Meldrum, N. U., and Roughton, F. J. W., *J. Physiol.*, **80**, 113 (1933).
2. van Goor, H., *Enzymologia*, **8**, 113 (1940).

3. Kiese, M., and Hastings, A. B., *J. Biol. Chem.*, **132**, 267 (1940).
4. Brinkman, R., *J. Physiol.*, **80**, 171 (1933).
5. Philpot, F. J., and Philpot, J. St. L., *Biochem. J.*, **30**, 2191 (1936).
6. Keilin, D., and Mann, T., *Biochem. J.*, **34**, 1163 (1940).
7. Leiner, M., *Z. vergl. Physiol.*, **26**, 416 (1938).
8. Roughton, F. J. W., and Booth, V. H., *Biochem. J.*, **40**, 319 (1946).
9. Stadie, W. C., and O'Brien, H., *J. Biol. Chem.*, **103**, 521 (1933).
10. Kiese, M., and Hastings, A. B., *J. Biol. Chem.*, **132**, 281 (1940).
11. Roughton, F. J. W., and Booth, V. H., *Biochem. J.*, **40**, 309 (1946).

THE THYROXINE-LIKE ACTIVITY OF COMPOUNDS STRUCTURALLY RELATED TO THYROXINE*

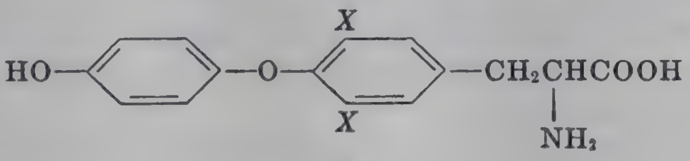
By EARL FRIEDEN† and RICHARD J. WINZLER

(*From the Department of Biochemistry and Nutrition, University of Southern California School of Medicine, Los Angeles*)

(Received for publication, March 27, 1948)

In the course of a search for compounds which might prove to be competitive inhibitors of thyroxine in its action on the peripheral tissues, we have initiated a study of the relationship between chemical structure and thyroxine-like activity of a number of thyroxine analogues.

Early attempts to relate chemical constitution to physiological activity have been summarized by Harington (1, 2) who concluded that the structural requirements for activity were best represented by (I), where *X* is a halogen and the alanine side chain may be functionally substituted. The report by Loeser and Trikojus (3) of appreciable thyroxine-like activity of thyroxamine, in agreement with the work of Abderhalden *et al.* (4) but contrary to that of Gaddum (5, 6), appeared to indicate that an intact alanine side chain may not be required for thyroxine-like activity. Later Niemann *et al.* (7, 8) suggested the importance of a quinoid resonance involving the phenolic hydroxyl group and the ether oxygen, the amino acid side chain possibly being involved in the incorporation into a peptide linkage. These considerations have led us to investigate the effect of other previously untested modifications of the side chain on physiological activity.



(I)

Materials

DL-Thyroxine[1] was used as the standard in all of the experiments reported. It was dissolved in a minimum amount of dilute sodium hydroxide and the solution adjusted to pH 8.0 ± 0.5 before being made up to volume.

---

* This work was aided by grants from the Committee on Research in Endocrinology of the National Research Council and by Eli Lilly and Company. Acknowledgment is made to the Hancock Foundation for providing facilities used in this work.

† Some of the data were taken from a thesis presented by Earl Frieden to the Graduate School of the University of Southern California in partial fulfillment for the requirements for the degree of Master of Science.

[1] We are indebted to Dr. Kenneth W. Thompson of Roche-Organon, Inc., Nutley 10, New Jersey, for a generous supply of DL-thyroxine.

3,5-Diiodo-L-tyrosine, m.p. 202–204°, was prepared from L-tyrosine by the method of Block and Powell (9). The possibility of *in vitro* conversion of diiodotyrosine to thyroxine was minimized in most cases by dissolving a neutral suspension of diiodotyrosine in dilute alkali and adjusting to pH 8 only a few minutes prior to the time of injection.

3,5-Diiodo-4-(3′,5′-diiodo-4′-hydroxyphenoxy)-benzoic acid, m.p. 255°, referred to as the benzoic acid analogue, was obtained as described by Harington and Barger (10). $C_{18}H_6O_4I_4$, calculated, I 69.2 per cent; found, 68.5 per cent. It was given as the sodium salt.

3,5-Diiodo-4-(3′,5′-diiodo-4′-hydroxyphenoxy)-DL-phenylglycine, the glycine homologue of thyroxine, was synthesized as described by Frieden and Winzler (11). It was administered in the same manner as DL-thyroxine.

N-Acetyl-DL-thyroxine, m.p. 210–214°, was prepared by the method of Kendall and Osterberg (12), with the corrections of Ashley and Harington (13). As expected, the compound gave a negative ninhydrin test and a positive Kendall reaction with nitrous acid. It was administered as the sodium salt at pH 8.0 ± 0.5.

N-Acetyl-3,5-diiodo-L-tyrosine, m.p. 198–200°, was synthesized according to Myers (14). This substance also gave a negative ninhydrin test and a positive Kendall reaction with nitrous acid and was administered as the sodium salt.

Methods

Amphibian Metamorphosis—The effect of thyroxine and its analogues on amphibian metamorphosis was followed by observing the diminution of total body length essentially according to the method of Gaddum (5). Groups of five larvae (*Bufo* sp.) were placed in 250 ml. glass dishes containing 200 ml. of tap water in which the test substance had previously been dissolved. The animals were measured with a micrometer on white oilcloth by stretching them out to their full length. In this way, animals could rapidly be classified according to size without injury. They were not fed during the course of the experiment, no decrease in length being observed for untreated controls under these conditions. Results with at least three different concentrations of DL-thyroxine administered to tadpoles, in addition to untreated controls, were observed along with the test substance in each experiment. Experiments were brought to a conclusion when a thyroxine control containing 0.25 γ per ml. revealed a 40 to 50 per cent reduction in length. 7 to 10 days were required when the tests were conducted at room temperature, 25° ± 2°. However, only 3 to 4 days were required when the assay was conducted in an incubator at 30.0° ± 0.5°.

A typical dose-response curve for thyroxine is given in Fig. 1. Considerable variation in response to thyroxine was observed in experiments carried out at different times, owing to fluctuations in temperature and to differences in the ages of the tadpoles.

Goiter Prevention Method—The goiter prevention method for the biological determination of DL-thyroxine or thyroxine-like activity in rats has been employed by Dempsey and Astwood (15), Reineke, Mixner, and Turner (16), and others. Groups of four to six albino rats of the University of Southern California strain, weighing between 100 and 200 gm., were used throughout these experiments. Over the 15 day assay period, the experimental animals were fed *ad libitum* with 0.3 per cent thiouracil incorporated in their stock diet. Daily intraperitoneal injections of the test compounds were given for 14 days, the injection volume usually being

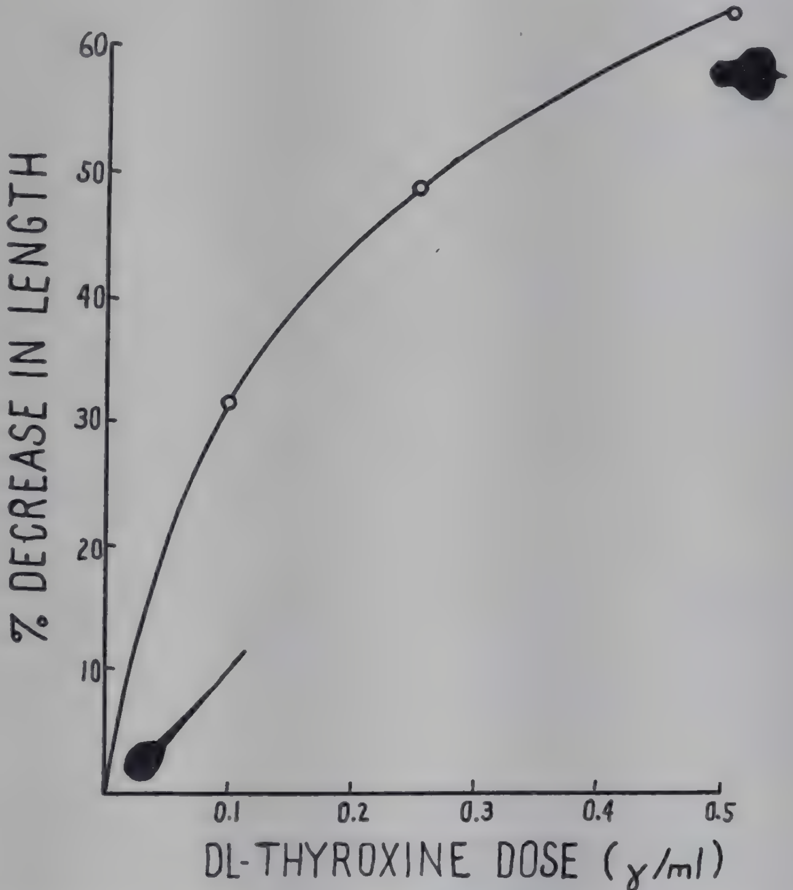


FIG. 1. DL-Thyroxine dose plotted against response in terms of per cent decrease in length in a typical experiment with larvae of *Bufo* sp. Groups of five tadpoles, 20 to 25 mm. in length, were incubated at 25° ± 3° for 7 days or at 30.0° ± 0.5° for 3 to 4 days. Initial and final lengths were determined to the nearest 0.5 mm. Contrast in body shape of the larvae with increasing DL-thyroxine concentration has also been indicated.

about 0.25 ml. per 100 gm. The animals were weighed and sacrificed on the 15th day. The thyroid glands were then removed and weighed to the nearest 0.1 mg. The room temperature was in the range of 24° ± 1.6°. DL-Thyroxine controls were used in each series of experiments. A typical standard curve showing the direct relationship between DL-thyroxine dose and thyroid weight response is shown in Fig. 2.

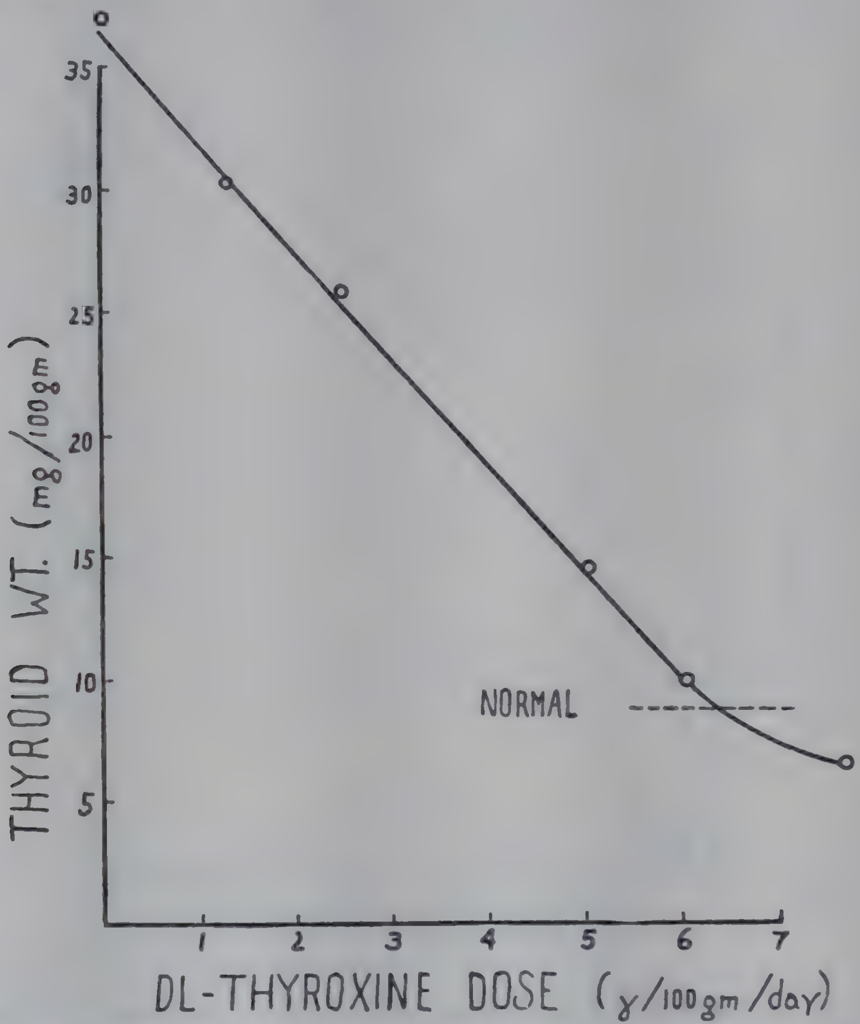


FIG. 2. Typical standard curve showing the effect of DL-thyroxine dose, in micrograms per 100 gm. of body weight, on the thyroid weight, in mg. per 100 gm., in thiouracil-fed rats. Groups of approximately six 150 to 200 gm. females were fed a 0.3 per cent thiouracil diet for 15 days, during which time they received daily intraperitoneal injections of the appropriate amount of DL-thyroxine.

RESULTS AND DISCUSSION

The results of the tests on the various compounds are summarized in Tables I and II, which show that 3,5-diiodo-L-tyrosine, the benzoic acid analogue, N-acetylthyroxine, and the glycine homologue of thyroxine exert thyroxine-like activity in both species.

It is evident from Table I that under the conditions of this experiment sodium iodide, even at 1.25 mg. per ml., had no influence on the body length of tadpoles. In longer term experiments, however, it has frequently been observed that iodide ion will accelerate the rate of metamorphosis of amphibian larvae. Concentrations of organic iodine containing compounds such as tetraiodophthalate and N-acetyldiiodotyrosine as high as 0.2 and 1.0 mg. per ml. respectively had no detectable influence on body length.

In Table I, diiodotyrosine is shown to exert a pronounced effect on the body length of tadpoles at levels of a few micrograms per ml. Thus, the thyroxine-like activity of diiodotyrosine is at least several hundred times greater in these experiments than an equivalent amount of inorganic iodine. The thyroxine-like action of 3,5-diiodo-L-tyrosine on the body length and shape of tadpoles ranged from 1 to 2 per cent of that of DL-thyroxine, since 25 γ per ml. of diiodotyrosine gave an effect equal to that of about 0.25 γ per ml. of thyroxine. It cannot yet be stated whether or not this activity is due to the conversion of diiodotyrosine to thyroxine in the incubation medium or in the organism. We have observed, however, that diiodotyrosine solutions kept at room temperature for some months show a steadily increasing thyroxine-like action, rising to as high as 6 per cent of that of thyroxine after 12 months of storage.

Table I also shows that the thyroxine-like effects of the glycine homologue and benzoic acid analogue of thyroxine on body length and shape of tadpoles are very high. Although the possibility that these compounds may be converted to thyroxine cannot be eliminated, we believe it more likely that the thyroxine-like action is inherent in the compounds themselves.

It is interesting that acetylation of the amino group of either thyroxine or diiodotyrosine markedly reduces their thyroxine-like action in Amphibia. Similar results have been previously reported for N-acetylthyroxine (Kendall (17), Swingle *et al.* (18)). This suggests that the acetylated compounds are either used inefficiently as such or are only poorly hydrolyzed to the free amino acids.

Table II shows that diiodotyrosine, the glycine homologue, and the benzoic acid analogue show definite thyroxine-like activity in the prevention of thiouracil goiters. This action is very definite, although relatively large amounts of the compounds are necessary to prevent thyroid enlargement completely. The prevention of thiouracil goiters implies a participation of these compounds directly or indirectly in the thyroid-pituitary axis, and need not necessarily indicate a thyroxine-like influence on peripheral tissue. Preliminary experiments on the effect of diiodotyrosine, N-acetylthyroxine, and the benzoic acid analogue on the basal metabolic rates of rats have indicated, however, that a metabolic stimulation also occurs with the administration of these compounds.

It is well known that iodide administration partially reduces the goitrogenic action of thiouracil in rats (19). However, in our experience, one experiment being shown in Table II, this effect has never exceeded 35 per cent restoration even at very high iodide levels. From the fact that the

TABLE I

Thyroxine-Like Activity of Compounds As Determined by Amphibian Metamorphosis

Compound	Dose	Per cent decrease in length	Per cent thyroxine-like activity*	Average thyroxine-like activity of compound
	γ per ml.			*per cent*
3,5-Diiodo-L-tyrosine (fresh)	5	9	0.5	
	15	25	2.0	
	25	45	1.0	
	50	64	1.0	
	25	33	1.0	
	50	53	1.0	1.0
Benzoic acid analogue	2.0	18	10	
	3.0	40	15	
	1.5	23	10	
	2.5	41	20	
	4.5	50	12	
	0.5	14	20	
	3.0	31	10	
	1.5	39	30	
Glycine homologue	4.5	65	20	16
	2.5	33	20	
	0.25	25	50	
	1.0	55	50	
	1.5	60	50	
	2.5	37	20	35
N-Acetyl-DL-thyroxine	5.0	10	2	
	12.5	37	3	
	12.5	42	3	
	25.0	55	2	2.5
N-Acetyl-3,5-diiodo-L-tyrosine	500	0	0	
	1000	0	0	
	2500	22	0.005	0.005
Potassium iodide	25	−6	0	
	125	(Toxic)		0
Sodium iodide	50	0	0	
	125	0	0	
	250	0	0	
	500	0	0	
	1250	0	0	0

---

* Estimated from the DL-thyroxine curve based on untreated controls and several DL-thyroxine levels observed simultaneously with the test substance.

effect of thiouracil on the weights of the thyroid gland can be completely negated by the administration of diiodotyrosine or the benzoic acid analogue, it is inferred that these compounds affect the thyroid-pituitary axis independently from the possible liberation of iodide. Similarly the significant effect of the glycine homologue at a level of 5 mg. per kilo is interpreted as a thyroxine-like action of this compound.

TABLE II

Thyroxine-Like Activity of Compounds As Determined by Goiter Prevention Method

Daily intraperitoneal injection	Daily dose	Thyroid weight	Decrease in thiouracil effect*	DL-Thyroxine equivalent†	Per cent DL-thyroxine activity
	mg. per 100 gm.	*mg. per 100 gm.*	*per cent*	γ	
3,5-Diiodo-L-tyrosine	2.5	10.6 ± 1.4‡	94	2.5	0.10
	15.0	17.8 ± 3.2	65	4.2	0.028
	25.0	10.8 ± 1.3	95	5.7	0.023
Benzoic acid analogue	0.25	30.4 ± 2.5	20	0.7	0.28
	1.0	23.8 ± 9.9	16	1.1	0.11
	1.0	18.4 ± 1.8	52	1.5	0.15
	5.0	7.3 ± 0.6	112	4.0	0.08
Glycine homologue§	0.50	20.9 ± 3.9	45	1.0	0.20
N-Acetyl-DL-thyroxine	0.0050	24.3 ± 5.1	43	1.5	30
	0.0125	13.1 ± 1.9	72	1.9	15
N-Acetyl-3,5-diiodo-L-tyrosine	5.0	20.6 ± 2.1	57	2.1	0.04
Potassium iodide	25.0	28.1 ± 3.9	32	1.9	<0.008

* Per cent decrease in thiouracil effect $= 100 \times (X - Y)/(X - N)$, where X is the thyroid weight of thiouracil controls, Y that observed with the test compound, and N that of normal controls (9.5 mg.).

† Estimated from the standard DL-thyroxine curve based on the thiouracil controls and one or more DL-thyroxine levels run simultaneously with the substance under test.

‡ Average deviation.

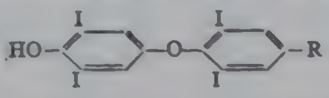
§ Mice were used as test animals for this experiment to permit testing of this compound at a sufficiently high level.

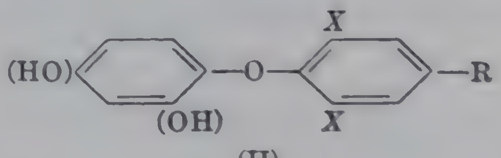
N-Acetylthyroxine showed one-sixth to one-third the activity of thyroxine when tested by the goiter prevention method. These data are in good agreement with those of Gaddum (6). The relatively greater activity of this compound and of N-acetyldiiodotyrosine in the rat compared to the tadpole is the only case that we have encountered in which compounds exert greater thyroxine-like activity in the higher species. The indication of a species difference in the utilization of N-acetylated thyroxine-like compounds may help to explain some of the earlier discrepancies on this point in the literature (6, 17, 18).

These findings permit some generalizations as to the importance of the side chain in the structural requirements for thyroxine-like activity. While the full side chain appears to be necessary for the maximum quantitative effect of a compound, this part of the molecule can nevertheless undergo considerable alteration without eliminating thyroxine-like activity. The data available on a number of compounds, involving side chain modifications, are presented in Table III. The series is not yet complete, but all compounds involving side chain alterations studied thus far have shown an appreciable thyroxine-like activity on Amphibia and a demonstrable activity on mammals. From this work and that of Niemann *et al.* (7, 8) we can generalize still further on the structural requirements for thyroxine-like activity. The major structural prerequisites, represented by (II), are as follows:[2,3] (1) an orthodihalogenophenolic-diphenyl ether configuration; (2) a hydroxy group either ortho or para to the ether oxygen; (3) a side chain which must include a functional group such as a carboxyl or an amino group or both. Maximum thyroxine-like activity is obtained

TABLE III

Effect of Side Chain Variation on Thyroxine-Like Activity

R of 	Per cent thyroxine-like activity		
	Amphibia	Mammalia	Bibliographic reference No.
COOH	16	0.16	This report
Ethylamine	50–100	50	(3)
Glycine	35	0.20	This report
Pyruvic acid		27	(22)
Alanine	100	100	
" N-acetyl	2.5	10–30	(6), this report
" N-glycyl		100	(6)
" N-alanyl		100	(6)



(II)

---

[2] The activity of 3,5-diiodo-L-tyrosine is the only important exception to II. This can be explained by assuming *in vivo* conversion to thyroxine. This is not unlikely in view of the work of von Mutzenbecher (20) and Dvoskin (21).

[3] Preliminary tests on the effects of 3,5-diiodo-4-(4′-hydroxyphenoxy)-aniline on Amphibia indicate that this compound is at least one-fourth as active as DL-thyroxine, lending further support to the suggestions made. Preparation of the corresponding tetraiodo aniline derivative is in progress.

when the side chain consists of an alanine group, the hydroxyl group is para to the ether linkage, and there are 4 iodine atoms occupying the 3,5 and 3′,5′ positions.

SUMMARY

Several compounds structurally related to thyroxine have been tested for thyroxine-like activity on amphibian metamorphosis and prevention of increase in the thyroid gland weights of thiouracil-fed rats. Demonstrable activity has been observed for the glycine homologue of thyroxine, a benzoic acid analogue of thyroxine, N-acetylthyroxine, and 3,5-diiodo-L-tyrosine in both tests. These findings indicate a lack of specificity of the side chain requirement for thyroxine-like activity.

It is suggested that the minimum structural requirements for thyroxine-like activity in Amphibia and mammals are the orthodihalogenophenolic-diphenyl ether configuration, a hydroxy group ortho or para to the ether oxygen, and a side chain which includes some functional group.

We are indebted to Miss Elizabeth V. Tukich for assistance in the goiter prevention assays.

BIBLIOGRAPHY

1. Harington, C. R., The thyroid gland; Its chemistry and physiology, London (1933).
2. Harington, C. R., *Fortschr. Chem. organ. Naturstoffe*, **2**, 103 (1939).
3. Loeser, A., and Trikojus, V. M., *Arch. Exp. Path. u. Pharmakol.*, **191**, 563 (1939).
4. Abderhalden, E., and Wertheimer, E., *Z. ges. exp. Med.*, **63**, 557 (1928).
5. Gaddum, J. H., *J. Physiol.*, **64**, 246 (1928).
6. Gaddum, J. H., *J. Physiol.*, **68**, 383 (1929–30).
7. Niemann, C., and Redemann, C. E., *J. Am. Chem. Soc.*, **63**, 1549 (1941).
8. Niemann, C., and Mead, J. F., *J. Am. Chem. Soc.*, **63**, 2685 (1941).
9. Block, P., Jr., and Powell, G., *J. Am. Chem. Soc.*, **65**, 1430 (1943).
10. Harington, C. R., and Barger, G., *Biochem. J.*, **21**, 169 (1927).
11. Frieden, E., and Winzler, R. J., *J. Am. Chem. Soc.*, in press.
2. Kendall, E. C., and Osterberg, A. E., *J. Biol. Chem.*, **40**, 265 (1919).
3. Ashley, J. N., and Harington, C. R., *Biochem. J.*, **23**, 1178 (1929).
4. Myers, C. S., *J. Am. Chem. Soc.*, **54**, 3718 (1932).
5. Dempsey, E. W., and Astwood, E. B., *Endocrinology*, **37**, 509 (1943).
6. Reineke, E. P., Mixner, J. P., and Turner, C. W., *Endocrinology*, **36**, 64 (1945).
7. Kendall, E. C., *Endocrinology*, **15**, 357 (1931).
8. Swingle, W. W., Helff, O. M., and Zwemer, R. L., *Am. J. Physiol.*, **70**, 208 (1924).
9. Charipper, H. S., and Gordon, in Harris, R. S., and Thimann, K. V., Vitamins and hormones, New York, **5**, 274–316 (1947).
0. von Mutzenbecher, P., *Z. physiol. Chem.*, **261**, 253 (1939).
1. Dvoskin, S., *Endocrinology*, **40**, 334 (1947).
2. Canzanelli, A., Guild, R., and Harington, C. R., *Biochem. J.*, **29**, 1617 (1935).

A FACTOR REQUIRED FOR THE GROWTH OF LEUCONOSTOC CITROVORUM*

By H. E. SAUBERLICH and C. A. BAUMANN

(*From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*)

(Received for publication, June 21, 1948)

During a survey of organisms suitable for the microbiological determination of alanine, it was found that *Leuconostoc citrovorum* 8081 failed to grow on a synthetic medium that was satisfactory for *Leuconostoc mesenteroides* P-60 and other assay organisms. However, the addition to the medium of certain crude extracts from liver resulted in a very rapid rate of growth of *L. citrovorum*, and the commercial liver preparation reticulogen (Lilly), active in Addison's pernicious anemia, proved to be effective in such small concentrations that the organism could be used for the microbiological determination of alanine and other amino acids in the presence of this supplement. Preliminary attempts were then made to determine the relationship between the unknown factor required by *L. citrovorum* and certain other growth factors, including the antipernicious anemia principle.

Methods

The organism, *Leuconostoc citrovorum* 8081, American Type Culture Collection, Georgetown University, Washington, D. C., was maintained on a yeast-peptone stab medium and was transferred every 2 to 4 weeks. Prior to use for assay purposes, the organism was grown in 10 ml. of a casein hydrolysate (Medium IV) (1) to which 0.2 per cent of yeast extract (Difco) had been added. A 20 to 24 hour culture was centrifuged twice and the cells diluted with 0.9 per cent NaCl to a standard degree of turbidity ($G = 65$ to 70 in a standard Evelyn tube, Filter 660). 1 drop of this diluted cell suspension was added to each assay tube, which also contained 1 ml. of the synthetic medium (Table I) plus the sample and water to make a total of 2 ml. The assay tubes were then incubated for 72 hours at 37° and the acid production measured by electrometric titration with 0.02 N NaOH.

EXPERIMENTAL

Response to Liver Preparations—The addition of the liver concentrate reticulogen, which according to the manufacturer contained 20 U. S. P.

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Committee on Growth, National Research Council, acting in behalf of the American Cancer Society.

units of the antipernicious anemia principle per ml., resulted in such rapid growth of the organism *Leuconostoc citrovorum* that a quantitative response could be measured turbidimetrically after only 10 hours (Fig. 1). A maximal response was observed when the medium contained 2.0 microliters of reticulogen per 10 ml. of medium as read, or 0.2 microliter per ml. However, when the incubation was prolonged for 72 hours, maximal acid production was observed with only 0.09 to 0.10 microliter of reticulogen per ml. of medium as titrated (0.2 microliter per 2 ml. tube, Fig. 2), and a half optimal response was observed when the medium contained only 0.025 microliter of reticulogen per ml. This latter half optimal response was designated as 1 "*citrovorum* unit" for the assay of other crude materials to obviate the necessity for a specific reference standard. The response of *L. citrovorum* to the active principle in liver was not altered by the addition

TABLE I

*Basal Medium VI**

	mg.		*mg.*
Amino acids		Salts A	
DL-α-Alanine	200	KH_2PO_4	600
L-Arginine	200	K_2HPO_4	600
L-Aspartic acid	100	Salts B	
L-Asparagine	400	$MgSO_4 \cdot 7H_2O$	200
L-Cysteine	50	$FeSO_4 \cdot 7H_2O$	10
L-Glutamic acid	300	$MnSO_4 \cdot 4H_2O$	20
Glycine	100	NaCl	10
L-Histidine	50	Purines and pyrimidines	
DL-Isoleucine	250	Adenine sulfate·$2H_2O$	10
DL-Leucine	250	Guanine hydrochloride	10
L-Lysine	200	Uracil	10
DL-Methionine	100	Xanthine	10
DL-Phenylalanine	100	Vitamins	
L-Proline	100	Thiamine hydrochloride	0.5
DL-Serine	50	Pyridoxine hydrochloride	1.0
DL-Threonine	200	Pyridoxamine hydrochloride	0.3
DL-Tryptophan	40	Pyridoxal hydrochloride	0.3
L-Tyrosine	100	Calcium *dl*-pantothenate	0.5
DL-Valine	250	Riboflavin	0.5
	gm.	Nicotinic acid	1.0
Glucose	25	*p*-Aminobenzoic acid	0.1
Sodium acetate	20	Biotin	0.001
Ammonium chloride	3	Folic acid	0.010
		Adjust to pH 6.8; add distilled H_2O to	500 ml.

* The designation, Medium VI, is used to distinguish this medium from those described in previous publications (1).

of large amounts of thiamine, pyridoxal, pyridoxamine, hydroxyproline, or the ash of yeast extract. Some production of acid, however, resulted when large amounts of crystalline folic acid were present in the medium. A response equivalent to 1 *citrovorum* unit was noted when 1.4 to 2 γ of folic acid were added per ml. of medium as titrated. The response of the organism to folic acid, however, differed qualitatively from that due to the factor in liver, since there was a marked delay in the growth of the organism given only the folic acid, and no visible growth was noted during the first 24 to 36 hours. Since the reticulogen preparation contained only 8.7 γ of folic acid per ml., the amount of folic acid in the 0.025 microliter of reticu-

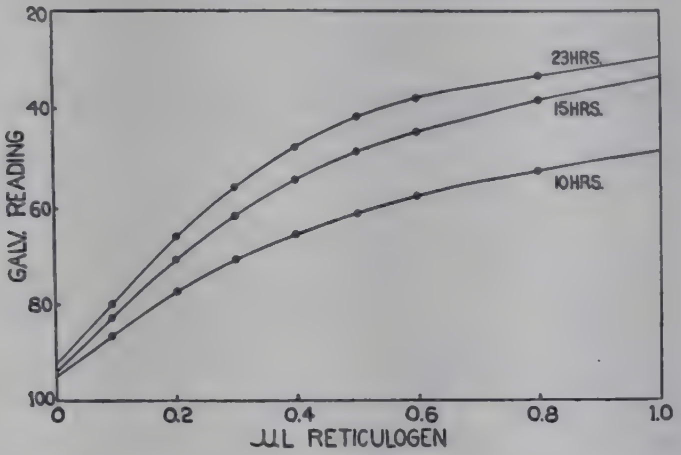


FIG. 1. Response of *Leuconostoc citrovorum* to graded amounts of reticulogen as determined by turbidity measurements after 10, 15, and 23 hours with the Evelyn colorimeter (660 mμ filter with an uninoculated tube set at 100 as the blank; 10 ml. volume assay).

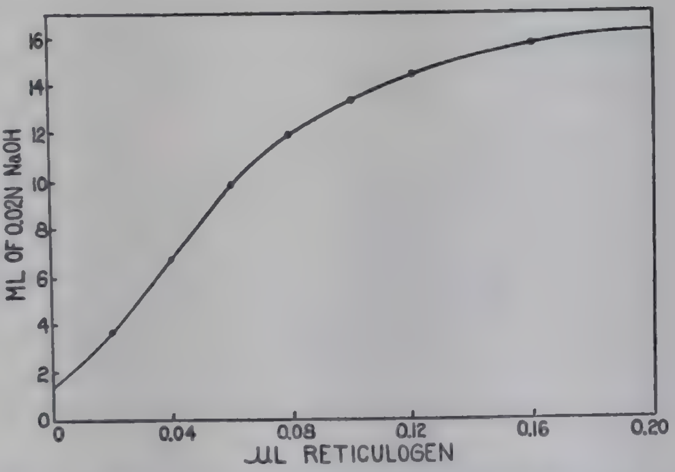


FIG. 2. Response of *Leuconostoc citrovorum* to graded amounts of reticulogen, as determined by the measurement of acid production after 72 hours (2 ml. volume assay).

logen required for half optimal growth supplied only 0.0002 γ of folic acid per ml. of medium. Thus it was obvious that the response of the organism to reticulogen could not be attributed to the folic acid present, for the amount of folic acid in the basal medium was 0.02 γ per ml. The data by Dunn and associates indicated that *L. citrovorum* 8081 grew fairly well on a medium containing 0.005 γ per ml. of a crude concentrate of folic acid (2), but that the organism failed to grow when the concentrate was replaced by crystalline folic acid (3).

TABLE II

Response of Leuconostoc citrovorum 8081 to Various Liver Concentrates Active against Pernicious Anemia

Sample No.	Liver extract*	U. S. P. units per ml.	*Citrovorum* units† per ml.
1	Lilly, Lot 360, reticulogen, enzyme-treated	20	41,900
2	" " 360, " untreated	20	40,000
3	" " 360 "	20	35,000
4	Sharp and Dohme, Lot 2505	15	34,000
5	Abbott, Lot E-1043	15	32,000
6	Lilly, Lot 370	2	20,200
7	Abbott, Lot E-1125	15	19,200
8	Lilly, Lot E-1181	Unknown	18,100
9	" " E-1203, per gm.	"	13,900
10	" " E-1204	"	11,720
11	" " 410	10	10,600
12	" " E-1180	Unknown	9,180
13	Abbott, Lot E-567	15	7,880
14	Lilly, Lot E-1108	20	6,710
15	Armour	10	4,930
16	Lederle, Lot 3641-34	10	4,880
17	Abbott, 95% alcohol extractives, per gm.	Unknown	2,140

* Sample 1, Lot 0030-442346, was treated with hog kidney enzyme in the manner used to liberate conjugated folic acid (4); Sample 3 was an unhydrolyzed sample of similar material, Lot 4250-418561; Samples 3 to 17 were obtained from Dr. W. R. Ruegamer, U. D. Register, Dr. C. A. Elvehjem, Dr. A. R. Robblee, C. A. Nichol, Dr. W. W. Cravens, J. J. Betheil, and Dr. H. A. Lardy of this department; Sample 6 was a relatively crude preparation high in solids; Sample 14 was a relatively purified preparation low in solids.

† The "*citrovorum* unit" was designated as the amount of substance required per ml. to give one-half maximal acid production in 72 hours by *Leuconostoc citrovorum* 8081 when grown on Basal Medium VI (Table I).

A series of preparations from liver ranged in activity from 2140 to 41,900 *citrovorum* units per ml. or gm. (Table II). Most of these were commercial products or experimental samples prepared as concentrates of the anti-pernicious anemia principle. All of the preparations which were active clinically were also active in promoting the growth of *Leuconostoc citrovorum*. Quantitatively, however, there was a fairly wide discrepancy between the potencies of the various preparations for the growth of *Leuconostoc citrovorum* and their reputed effectiveness for pernicious anemia patients. The most active preparation for the organism was a sample of the liver concentrate, reticulogen, which had been treated with hog kidney enzymes for 24 hours at pH 4.5 in a citrate-phosphate buffer. This treatment is effective in the release of folic acid from its conjugate (4). This preparation contained 41,900 *citrovorum* units per ml. (Table II, Sample 1), whereas an unhydrolyzed aliquot of the same preparation, reticulogen (Table II, Sample 2), contained 40,000 *citrovorum* units per ml. The hydrolytic procedure therefore failed to increase the activity of the preparation by more than 5 per cent. Since Sample 2 contained 20 U. S. P. (clinical) units of the antipernicious anemia factor and 40,000 *citrovorum* units per ml., 1 U. S. P. unit of this preparation contained 2000 *citrovorum* units. Essentially the same relationship was observed in a second reticulogen preparation, Sample 3, and in liver extracts Samples 4 and 5, in which 15 U. S. P. units per ml. were equivalent to 34,000 and 32,000 *citrovorum* units respectively (Table II). However, two other preparations from liver containing 15 U. S. P. units per ml. (Samples 13 and 7) were found to contain only 7880 and 19,200 *citrovorum* units per ml. The most active preparation for the organism in relation to reputed clinical potency was a crude preparation relatively high in solids in which 2 U. S. P. units corresponded to 20,200 *citrovorum* units (Table II, Sample 6), a ratio of 1:10,100. On the other hand, a highly purified preparation low in solids, Sample 14, which was reputed to contain 20 U. S. P. units per ml., was found to contain only 6700 *citrovorum* units per ml., a ratio of only 1:335. The ratio of clinical to microbiological response for all the samples was thus found to vary over a 30-fold range. This result is suggestive of the experience of Rickes and associates (5) with *Lactobacillus lactis* Dorner, in which the ratios of clinical to microbiological potencies of products containing 15 U. S. P. units per ml. varied over a 12-fold range.

Since the extracts consisted largely of crude materials, several possibilities are suggested: (*a*) that related compounds were present in preparations such as Sample 13 or 14, which were active clinically but were inactive microbiologically; (*b*) that part of the active principle occurred in a bound form not available to the microorganism and not released by hog kidney enzymes; or (*c*) that more than one factor is needed by the organism. A lack of precision in the results of clinical assays could also have been partly responsible for the discrepancies between the two types of biological responses. Incidentally, in the assay of representative liver preparations results essentially similar to the values obtained titrimetrically were also obtained with turbidimetric measurements after 10 to 15 hours. In the assay of folic acid for *citrovorum* units, however, turbidimetric assays yielded a value of zero, due to the delay in the growth response.

TABLE III

Growth of Leuconostoc citrovorum 8081 on Medium VI Fortified with Various Substances

Sample No.	Substance added*	*Citrovorum* units
18	Fish solubles (80% ethyl alcohol extract) per ml.	179
19	" " (BuOH-soluble) per ml.	137
20	" " (" pH 3) per ml.	132
21	" " (BuOH-insoluble) per ml.	24
22	" " (") " "	29
23	" " (BuOH-soluble, pH 10) per ml.	16
24	" " (" " 7) " "	16
25	Yeast extract (Difco) per gm.	4720
26	Bacto-peptone " " "	3240
27	Rice-bran concentrate (Vitab) per gm.	625
28	Folic acid (at 1 γ per ml. medium) per mg.	480
	" " (" 2" " " ") " "	1540
29	Formyl folic acid (at 4 γ per ml. medium) per mg.	470
30	Milk (pasteurized) per ml.	Very slight
31	Thymine (at 10–300 γ per ml. medium)	Inactive
32	Xanthopterin (at 2–60 γ per ml. medium)	"
33	Leucopterin (" 2–60 " " " ")	"
34	Yeast extract	"
35	Thymine desoxyriboside (at 0.4 γ per ml. medium) per mg.	2720

* Samples 18 to 24 were obtained from Dr. A. R. Robblee, C. A. Nichol, Dr. W. W. Cravens, and Dr. C. A. Elvehjem of this department. The preparation of these samples and their activities in chickens have been published (6, 7), or described in a thesis entitled "Studies on unidentified chick growth factors," Dr. A. R. Robblee, University of Wisconsin (1948). Sample 29 was obtained from J. J. Betheil and Dr. H. A. Lardy, Sample 31 from H. P. Broquist and Dr. E. E. Snell, Sample 32 from B. H. Olson and Dr. W. H. Peterson, Sample 33 from Dr. A. R. Robblee, C. A. Nichol, Dr. W. W. Cravens, and Dr. C. A. Elvehjem, Sample 34 (8) from W. L. Williams and Dr. E. E. Snell, and Sample 35 from Dr. E. E. Snell.

Chick Factors—Robblee and associates (6, 7) have found that a factor in certain crude materials is needed for the optimal growth of chicks fed all of the known vitamins available in pure crystalline form. The active principle is present in concentrates of fish solubles and in certain liver preparations, including reticulogen. Assays of various preparations from fish solubles, obtained from Messrs. Robblee, Nichol, and Elvehjem of this department, indicated that most of the preparations were only slightly or moderately active for *Leuconostoc citrovorum* (Table III). A preparation insoluble in butyl alcohol, Sample 21, which was very active in the chick,[1] contained only 24 *citrovorum* units per ml.; on the other hand, Sample 19, a fraction soluble in butyl alcohol, contained 137 units per ml. for the organism, but was inactive for the chicken.[1] Thus it would appear that the factors responsible for the respective responses in the two species are not identical. This is also suggested by the quantitative responses of the two species to the liver extracts Samples 5 and 13, respectively; the former was inactive in the chick assay,[1] although it proved to be one of the most potent concentrates of the *citrovorum* factor thus far encountered. On the other hand, Sample 13, which was very active in the chicken,[1] was only one-fourth as active as Sample 5 for the microorganism.

The factor required by *Leuconostoc citrovorum* appears to be different from the unknown principle required by a special strain of *Lactobacillus bulgaricus* (8). A preparation from yeast[2] that was very active for the latter organism[3] proved to be inactive for *L. citrovorum*. Furthermore, the factor required by *L. citrovorum* did not appear to coincide with the principle required for the optimal growth of hyperthyroid rats (9) as indicated in preliminary assays. Sample 13, which was highly active in the rat,[4] contained only 7880 *citrovorum* units per ml. as contrasted to Preparation 5, which was relatively inactive in the rat[4] but contained 32,000 *citrovorum* units per ml. (Table II).

Crude materials which stimulated the growth of *Leuconostoc citrovorum* included yeast extract, Bacto-peptone, and a rice-bran concentrate (Vitab), while milk contained very slight activity. Formyl folic acid[5] proved somewhat less active than folic acid itself, whereas xanthopterin, leucopterin, and thymine, when added at levels of 2 to 60 γ per ml., 2 to 60 γ per ml., and 10 to 300 γ per ml., respectively, were inactive for *Leuconostoc citrovorum*.

A sample of thymine desoxyriboside[2] stimulated the initial growth of *Leuconostoc citrovorum* 8081 in concentrations up to 0.6 γ per ml., but a further increase in the concentration of the riboside up to 2.0 γ per ml. failed to increase turbidity further. The maximal turbidity reached in 16 hours by the cultures containing the riboside was less than one-fourth that reached by cultures containing reticulogen. An intermediate concentration of the thymidine, 0.1 γ per ml., stimulated growth equivalent to that observed when 0.01 microliter of reticulogen was present per ml. If the latter response were due only to thymidine, this would indicate a thymidine content of 10 per cent in the solids of reticulogen.

---

[1] Personal communication with A. R. Robblee, C. A. Nichol, W. W. Cravens, and C. A. Elvehjem.

[2] We are indebted to W. L. Williams and E. E. Snell for this preparation.

[3] Personal communication with W. L. Williams and E. E. Snell.

[4] Personal communication with U. D. Register, W. R. Ruegamer, and C. A. Elvehjem.

[5] Obtained from J. J. Betheil and H. A. Lardy.

A similar result was obtained when the relative activities were based on the production of acid after 72 hours. But whereas increasing concentrations of reticulogen resulted in marked increases in the amounts of acid produced, further increases in the concentration of thymidine stimulated further acid production only slightly, with the result that the amounts of acid produced by cultures containing 1.0 γ of thymidine per ml. corresponded to an apparent thymidine concentration of 23 per cent in the solids of the liver concentrate. Thus, since the response of *Leuconostoc citrovorum* 8081 to thymidine differed qualitatively from that to the liver concentrate, it was concluded that some other active agent or agents must have been present in the latter preparation.

DISCUSSION

The fact that a 30-fold variation was encountered between U. S. P. units of the antipernicious anemia factor and the *citrovorum* units found in various preparations of liver might suggest that there is no relationship whatever between the antipernicious anemia principle and the factor or factors stimulating the growth of *Leuconostoc citrovorum.* However, when *Lactobacillus lactis* Dorner was used as the assay organism for products containing 15 U. S. P. units of the antipernicious anemia factor per ml., the ratios of clinical to microbiological units varied over a 12-fold range (5). Nevertheless, this organism was used successfully as an aid in the isolation of vitamin B_{12}. Analyses with *L. citrovorum* of Samples 4, 5, 7, and 13 (Table II), preparations containing 15 U. S. P. units per ml., indicated that the ratio of clinical to *citrovorum* units varied over only about a 4-fold range. Thus it is possible that *L. citrovorum* may prove to be superior to *Lactobacillus lactis* Dorner as an assay organism for the antipernicious anemia factor, although this cannot be determined until the details for the use of the latter organism are revealed. In any event, an exact correlation between the results of microbiological methods and those of clinical assays should not be expected, in view of the variations inherent in clinical responses.

Another reason for suspecting a metabolic connection between *Leuconostoc citrovorum* and pernicious anemia patients is the peculiar partial response of both to high levels of folic acid. High levels of this vitamin produce a partial remission of the symptoms of the disease without, however, curing all the symptoms benefited by crude concentrates from liver (10, 11). Similarly, high amounts of folic acid stimulate the growth of *Leuconostoc citrovorum* but in a delayed manner, qualitatively different from that observed with the best preparations from liver. One might, therefore, postulate a similarity in structure between folic acid and the natural agents active in the organism and the pernicious anemia patient. Another possibility is that folic acid is necessary for the synthesis of the unknown principles.

SUMMARY

1. *Leuconostoc citrovorum* 8081 failed to grow on a synthetic medium adequate for the growth of *Leuconostoc mesenteroides* P-60 and other common assay organisms. Growth was rapid in the presence of certain concentrates from liver, peptone, or yeast extract.

2. All liver preparations known to be active against pernicious anemia were also active for *Leuconostoc citrovorum*, but a 30-fold variation was found between preparations in the ratios of microbiological activity to reputed clinical potency.

3. The factor required by *Leuconostoc citrovorum* did not appear to be identical with the factor in fish solubles required by the chick, with the factor in liver required for the optimal growth of hyperthyroid rats, nor with the factor in yeast required by a strain of *Lactobacillus bulgaricus*.

4. High amounts of folic acid stimulated maximal acid production by *Leuconostoc citrovorum*, provided the culture was incubated for 72 hours. Qualitatively, however, the growth of the organism was much slower in the presence of high amounts of folic acid than when small amounts of liver preparations were present in the medium.

BIBLIOGRAPHY

1. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, **166**, 417 (1946).
2. Dunn, M. S., Shankman, S., Camien, M. N., and Block, H., *J. Biol. Chem.*, **168**, 1 (1947).
3. Camien, M. N., Dunn, M. S., and Salle, A. J., *J. Biol. Chem.*, **168**, 33 (1947).
4. Bird, O. D., Bressler, B., Brown, R. A., Campbell, C. J., and Emmett, A. D., *J. Biol. Chem.*, **159**, 631 (1945).
5. Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, **107**, 396 (1948).
6. Nichol, C. A., Robblee, A. R., Cravens, W. W., and Elvehjem, C. A., *J. Biol. Chem.*, **170**, 419 (1947).
7. Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *J. Biol. Chem.*, **173**, 117 (1948).
8. Hoff-Jørgensen, E., Williams, W. L., and Snell, E. E., 4th International Congress for Microbiology, Abstracts of communications, Copenhagen, July 20–26, 196 (1947).
9. Betheil, J. J., Wiebelhaus, V. D., and Lardy, H. A., *J. Nutr.*, **34**, 431 (1947).
10. Heinle, R. W., Dingle, J. T., and Weisberger, A. S., *J. Lab. and Clin. Med.*, **32**, 970 (1947).
11. Vilter, C. F., Vilter, R. W., and Spies, T. D., *J. Lab. and Clin. Med.*, **32**, 262 (1947).

CORRECTIONS OF PUBLISHED ELECTROPHORETIC MOBILITIES OF ADRENOCORTICOTROPIC AND PARATHYROID HORMONES

By ALFRED E. WILHELMI and GEORGE SAYERS

*(From the Department of Physiological Chemistry, Yale University, New Haven,
and the Department of Pharmacology, University of Utah
School of Medicine, Salt Lake City)*

(Received for publication, July 8, 1948)

A recent check-up of the electrophoresis apparatus in use at the Laboratory of Physiological Chemistry at Yale has revealed that the milliammeter of the power supply is shunted, so that the actual current delivered by the power supply is 3.4 times the current indicated on the meter. Since the direct milliammeter readings were used in the calculations of electrophoretic mobilities of the adrenocorticotropic hormone, published by Sayers, White, and Long (1), and of the components of the parathyroid hormone preparation described by L'Heureux, Tepperman, and Wilhelmi (2), the values obtained were all too high by a factor of 3.4. The corrected values are briefly summarized as follows:

For adrenocorticotropic hormone (1):

Acetate buffer, ionic strength 0.1, pH 3.26 = $+7.1 \times 10^{-5}$ cm.^{2} volt^{-1} sec.$^{-1}$
" " " " 0.13, " 4.13 = $+2.5 \times 10^{-5}$ " " "
Phosphate " " " 0.2, " 6.37 = -7.1×10^{-5} " " "
" " " " 0.2, " 7.95 = -8.3×10^{-5} " " "

For parathyroid hormone (2), in acetate buffer, ionic strength 0.1, pH 3.50; fast component = $+8.8 \times 10^{-5}$ cm.^{2} volt^{-1} sec.$^{-1}$; slow component = $+6.8 \times 10^{-5}$ cm.^{2} volt^{-1} sec.$^{-1}$.

If one makes allowance for the differences in ionic strengths of the buffers used by the two groups of investigators, the corrected values for the electrophoretic mobilites of adrenocorticotropic hormone reported by Sayers *et al.* are now in substantial agreement with those reported by Li, Evans, and Simpson (3).

The authors apologize for their lack of complete understanding of their instrument. They hope that the foregoing corrections may help to avoid misunderstanding and confusion.

SUMMARY

The values for the electrophoretic mobilities of the adrenocorticotropic hormone published by Sayers *et al.* and for those of a parathyroid hormone preparation published by L'Heureux *et al.* have been found to be too large

by a factor of 3.4. When this correction is applied to the mobilities of the adrenocorticotropic hormone, the new values are seen to be in substantial agreement with those reported for this hormone by Li, Evans, and Simpson.

BIBLIOGRAPHY

1. Sayers, G., White, A., and Long, C. N. H., *J. Biol. Chem.*, **149,** 425 (1943).
2. L'Heureux, M. V., Tepperman, H. M., and Wilhelmi, A. E., *J. Biol. Chem.*, **168,** 167 (1947).
3. Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, **149,** 413 (1943).

PREPARATION AND SOME PROPERTIES OF HYALURONIC ACID FROM UMBILICAL CORD OF THE PIG*

By ARCHIE E. FOLLETT†

(*From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts*)

(Received for publication, June 12, 1948)

Within the last 13 years hyaluronic acid has been isolated from a variety of sources (1): umbilical cord, vitreous humor, synovial fluid, tumors, and hemolytic streptococci. These mucopolysaccharides all have essentially the same chemical composition, equimolecular portions of acetylglucosamine and glucuronic acid, but vary greatly in viscosity. Hyaluronic acid was first isolated by Meyer and Palmer (2) in 1934 from bovine vitreous humor. Human umbilical cord was shown by them (3) to be a rich source of hyaluronic acid. Recently Hadidian and Pirie (4) have prepared from this same source a product more viscous than any hitherto obtained. However, the unbilical cord of no species other than the human has yet been investigated as a source of hyaluronic acid. It was the purpose of this work to investigate pig umbilical cord as a source and examine the properties of the hyaluronic acid derived.

EXPERIMENTAL

Fractionation—Fresh frozen pig umbilical cords were used as the starting material in this work. In previous work, human umbilical cords were stored in acetone 1 to 6 weeks before processing. In this work, the effect of this acetone storage on the extraction procedures was studied by extracting a portion of fresh frozen pig umbilical cords as received, while a second portion was set aside in acetone for 6 weeks before the extraction of hyaluronic acid. The method of extracting hyaluronic acid was essentially the fractionation technique described by Hadidian and Pirie (4). The umbilical cords were cut up and extracted with water and the residue was ground and extracted with 0.1 M sodium chloride. At this point in the procedure an innovation was introduced; the residue from the sodium chloride treatment was extracted with boiling water. The residue was suspended in 0.01 M hydrochloric acid (the pH readjusted to 2 when necessary) and

---

* This investigation was aided by a grant from the L. Farber Company of Worcester, Massachusetts. Pig umbilical cords were obtained through the courtesy of G. D. Searle and Company, Chicago, Illinois.

† Present address, Research Department, Phillips Petroleum Company, Phillips, Texas.

digested at 37° with pepsin, followed by trypsin at pH 7.6. Hyaluronic acid was precipitated from the respective fluids in the following manner: Hydrochloric acid was added and formed a "mucin clot" which was removed; $1\frac{1}{3}$ volumes of ethanol were added to the acid fluid and the precipitate removed; solid ammonium sulfate was added to the alcoholic supernatant fluid with vigorous mechanical agitation, and on standing the system separated into two layers with hyaluronic acid appearing at the interface. A precipitate was not always obtained at each step in the procedure.

In this manner, both the fresh frozen pig umbilical cords and the acetone-stored cords were extracted. For purposes of comparison, human umbilical cords (acetone-stored) were extracted according to the above procedure. Supplementary work was done with pig cords by grinding fresh frozen material, and the entire mass incubated with pepsin at 37°, followed by trypsin. This digested fluid was put through the acid-alcohol-ammonium sulfate fractionation.

Purification—The mucin clots were partially fractionated by precipitation with alcoholic potassium acetate from alkaline solution, as in McClean's (5) procedure, or incubation with pepsin and trypsin, the digestion procedure being more satisfactory usually.

Several of the preparations referred to in Table I were made by removing the protein by shaking with a mixture of 1 part chloroform and 2 parts amyl alcohol (volume per volume) according to the Sevag technique (6). The hyaluronic acid was precipitated from acid solution by $1\frac{1}{3}$ volumes of ethanol, then suspended in a smaller volume of water and dialyzed.

Chemical Analyses—The nitrogen content was determined by the micro-Kjeldahl procedure. The acetyl content was measured by hydrolyzing a 1 ml. sample in 2.5 N H_2SO_4 at 100° for 75 minutes, steam-distilling in the apparatus described by Markham (7), and titrating the distillate with N/75 NaOH. The glucosamine determinations were made by the method of Elson and Morgan (8) on material that had been hydrolyzed for 6 to 8 hours in 5 N HCl at 100° and then evaporated to dryness in a vacuum desiccator over a moistened caustic. All of these analyses were made on aliquots of dialyzed solutions whose concentration had been determined by drying at 100° for 2 hours and cooled in a desiccator. The values refer to the free acid and not to a salt.

Viscosity Measurements—Viscosity measurements were made in an Ostwald viscosimeter with a capillary 9 cm. long and having a flow time of about 30 seconds for 4 ml. of distilled water. This volume was used in all the experiments reported, and all the measurements were made at 25°. The standard salt concentrations used throughout were 0.05 M NaCl and 0.05 M phosphate buffer at pH 7.0. This concentration of salt and buffer

TABLE I

Analyses of Mucopolysaccharides Isolated from Umbilical Cord

Extraction	Sample No.	Treatment	Nitrogen	Glucosamine	Acetyl	Relative viscosity	Half time
A. Pig (fresh frozen)							
			per cent	*per cent*	*per cent*		*sec.*
0.1 M NaCl	1	Interfacial	12.1			1.01	
Hot water	2	"	5.8			1.02	
Pepsin-trypsin digest	3	Portion, 1.33 vols. ethanol	5.5	20.0	6.6	1.04	
" "	4	Bulk, 1.33 vols. ethanol	5.3		7.9	1.06	
" "	5	Samples 3 and 4 combined, Sevag purification	3.5	31.4	7.7	1.08	
Mucin clots	6	Alcoholic potassium acetate purification	3.7	23.4	6.6	1.03	
" "	7	Sample 6, Sevag purification	3.1	34.6	7.9	1.03	
Yield: gross, 13.2%; corrected to 3.6% N, 5.4%							
B. Pig (fresh frozen; acetone-stored)							
0.1 M NaCl	10	Mucin clot, alcoholic CH_3-COOK purification	6.5			1.03	
0.1 " "	11	1.33 vols. ethanol	5.2	31.5	8.1	1.31	760
0.1 " "	12	Sample 11, Sevag purification	3.2	37.9	10.2	1.38	482
0.1 " "	13	Interfacial	9.5			1.03	
Hot water	14	" digested, 1.33 vols. ethanol	3.5	34.9	8.5	1.15	920
Pepsin-trypsin digest	15	Mucin clot	10.6			1.01	
" "	16	1.33 vols. ethanol	5.4	34.1	6.5	1.07	
" "	17	Sample 16, Sevag purification	3.2	31.3	9.0	1.08	
Yield: gross, 5.3%; corrected to 3.6% N, 3.3%							
C. Pig (fresh frozen)							
Pepsin-trypsin digest	20	1.33 vols. ethanol	5.6	30.6	8.1	1.17	
" "	21	Sample 20, Sevag purification	3.6	29.0	7.8	1.15	957
Yield: gross, 10.0%; corrected to 3.6% N, 6.6%							

TABLE I—*Concluded*

Extraction	Sample No.	Treatment	Nitrogen	Glucosamine	Acetyl	Relative viscosity	Half time
D. Human (acetone-stored)							
			per cent	*per cent*	*per cent*		*sec.*
Initial water	30	Interfacial	3.2				
0.1 M NaCl	31	"	3.3	30.8	10.2	1.53	285
0.1 " "	32	Sample 31, Sevag purification	3.0	41.8	11.4	1.49	206
Hot water	33	Interfacial	14.8			1.02	
Initial water	34	1.33 vols. ethanol	5.0			1.59	
" "	35	Sample 34, Sevag purification	3.4	43.0	8.6	1.51	293
Initial water, 0.1 M NaCl, hot water combined	36	Mucin clots digested, HCl ppt.	2.6	11.2	5.0	1.03	
" "	37	Mucin clots digested, 1.33 vols. ethanol	4.0	35.8	7.7	1.23	1244
" "	38	Sample 37, Sevag purification	3.4	34.4	9.2	1.20	735
0.1 M NaCl, hot water combined	39	1.33 vols. ethanol	3.1			1.93	
" "	40	Sample 39, Sevag purification	2.8	41.7	11.2	1.87	146
Yield: gross, 4.9%; corrected to 3.6% N, 4.4%							

was used, since it was the one found by Hadidian and Pirie (9) to give optimum results. The term "relative viscosity" refers to the ratio of flow time of the test solution to the flow time of a solution with the same salt concentration. For measuring the relative viscosity of hyaluronic acid preparations a standard concentration of 0.3 gm. per liter was adopted.

The rate at which hyaluronic acid preparations lost viscosity with bull testis hyaluronidase was measured by incubation at 25° with the enzyme in the standard ionic environment. The time required for the viscosity to fall half way from the initial to the presumed final value was taken as a measure of the rate of enzyme action. Half time measurements are customary in studies of this enzyme. Partially purified bull testis enzyme (Schering) was used in all of these experiments at a concentration of 50 γ in 4 ml. of test solution.

Preparation of Inhibitors

Nitration—A few hyaluronic acid preparations were nitrated to test their inhibition of hyaluronidase hydrolysis of hyaluronic acid. The nitration procedure used was that described by Hadidian and Pirie (9). Both the acid-insoluble and the acid-soluble portions were tested for their inhibitory effect at a concentration of 0.03 gm. per liter. The nitrogen content was determined by the micro-Kjeldahl method modified to include the nitrogen of nitrates.

Acetylation—Preparations from pig cord were acetylated to study their inhibitory effect on the hyaluronidase-hyaluronic acid system. The acetylation procedures used were the two described by Hadidian and Pirie (9). In the sulfuric acid method acetylation was allowed to proceed for 30 to 45 minutes at 27°. Acetylation was continued for 2 hours at 20° with the pyridine method. The acetyl content was determined by the method used above for hyaluronic acid preparations and included both O-acetyl and N-acetyl.

Results

Umbilical Cord—Pig umbilical cord proved to be a rich source of hyaluronic acid, nearly as rich as human cord. The yield of hyaluronic acid obtained from acetone-stored material was 3.3 per cent of the dry weight of the pig cord as compared with 4.4 per cent for human cord (these values corrected to 3.6 per cent nitrogen).

The most striking difference observed (Table I) for the series of pig cord preparations was in the relative viscosity; all fresh pig cord preparations were very low in viscosity, whereas one product in particular from defatted pig cord (Sample 12) with a relative viscosity of 2.3, calculated to a concentration of 1 gm. per liter, compared very favorably with the better preparations from various sources reported by other investigators. A compilation of these has been made by Hadidian and Pirie (4). The values for the chemical constituents of preparations from fresh frozen cords were lower than those from defatted cords. It was also noted that for pig cord in general the viscous preparations contained 8.5 to 10.2 per cent acetyl, but preparations made by enzymic digestion of residues had lower acetyl contents, as low as 6.5 per cent. There was a comparable deficiency in the glucosamine content. Viscous products contained 35 to 38 per cent, whereas the non-viscous had less than 35 per cent. The nitrogen values were in the range of 3.1 to 3.5 per cent.

The fresh frozen pig cords which were ground and subjected to pepsin-trypsin digestion without prior treatment produced the highest yield of hyaluronic acid. Since no fractionation was involved in this procedure, the relative viscosity and acetyl and glucosamine values were midway between those for the viscous and non-viscous preparations obtained previously. This was, of necessity, anticipated, as this preparation represented the average of the heterogeneous hyaluronic acid fractions present. As has been observed in previous work precipitating hyaluronic acid, the pepsin-trypsin digestion solution yielded no mucin clot with concentrated HCl and no interfacial material separated, but the precipitate was obtained with alcohol. This crude material was not excessively contaminated with protein; the nitrogen content was 5.6 per cent which dropped to 3.6 per cent on purification by the Sevag technique followed by dialysis. The half time of 957 seconds for Sample 21 was about what would be expected from a preparation having a relative viscosity of 1.15.

Inhibitors—Three pig cord products, Samples 5, 12, 21, were nitrated; both the acid-insoluble and the acid-soluble fractions were tested for their inhibiting effect on the hyaluronidase-hyaluronic acid system. Sample 5 was non-viscous, Sample 12 the most viscous pig product, and Sample 21 intermediate. The acid-soluble fractions of Samples 5 and 12 were combined before measuring their inhibition. This was done because of the similarity in the inhibition given by their acid-insoluble products. The values obtained for all of the inhibitors with a brief description of the derivation of each are given in Table II. The acid-soluble products were found to be better inhibitors than the acid-insoluble fractions. Of these nitrated products, the one derived from the non-viscous preparation gave the greatest inhibition. Nitrogen analyses (modified micro-Kjeldahl) made on the nitrated products from Sample 21 indicated that the acid-insoluble material was nitrated to the extent of one —NO_2 group, and the acid-soluble one-half an —NO_2 group. The inhibition found for these nitrated pig cord preparations was of about the same order of magnitude as that reported by Hadidian and Pirie (9) for their inhibitors made from human cord products.

TABLE II

Inhibitors, Nitrated and Acetylated

In these experiments the inhibitor was measured at a final concentration of 0.03 gm. per liter. The half time was found in the usual manner, and the amount of inhibition given in the right-hand column was derived by dividing the half time found in the presence of the inhibitor by that found in the control experiment with the enzyme and substrate alone.

Description of inhibitor	Inhibition
Sample 5. Nitrated, acid-insoluble	2.1
" 12. " "	1.6
Samples 5, 12. Nitrated separately, acid-soluble fractions combined	4.8
Sample 21. Nitrated, acid-insoluble, 5.9% N	1.5
" 21. " acid-soluble, 4.7% N	2.1
" 21. Acetylated, sulfuric acid method, 18.2% acetyl	1.3
" 21. " pyridine method, 18.4% acetyl	1.2

One hyaluronic acid preparation from pig cord was acetylated by both the sulfuric acid catalyst method and the pyridine method; by each method the resulting product contained about 18 per cent acetyl. Acetylated products were found to be poorer inhibitors than the nitrated acid-soluble products.

DISCUSSION

Hyaluronic acid can be extracted from pig cord and purified by the same methods that have been used for human umbilical cord. The products prepared from pig cord contained practically the same proportion of nitrogen, acetyl, and glucosamine as preparations from human cord. The general trend in the physical and chemical properties noted by Hadidian and Pirie (4) for human cord preparations was found here to be the same for pig cord; *i.e.*, mucin clots had the lowest viscosity with material from pepsin-trypsin-digested extracts intermediate between those and the viscous products, and the non-viscous preparations contained less than the theoretical percentage of both acetyl and glucosamine. Preparations of hyaluronic acid from pig cord were hydrolyzed by the enzyme hyaluronidase (testis). Nitrated and acetylated pig preparations were found to inhibit the hyaluronidase-hyaluronic acid system to the same extent as inhibitors made from human cord.

In comparing the physical and chemical properties of hyaluronic acid preparations from pig umbilical cord with those from human cord, the most obvious difference was found in the relative viscosity; the human cord products were appreciably more viscous. However, the half time of products from both sources was found to be inversely proportional to the relative viscosity. The acetyl and glucosamine content of human preparations ranged, in general, higher than in pig preparations. These differences in properties observed may be attributable to a species difference.

The quality of pig cord preparations extracted was enhanced by acetone storage prior to processing for hyaluronic acid. Apparently a protein denaturation process was involved which decreased the solubility of the proteins since the crude products from the fresh frozen pig cords were contaminated with protein to a far greater extent than those from the defatted pig cords. The innovation of adding a boiling water treatment to the residue from the 0.1 M NaCl extraction in the procedure was of no particular efficacy. A substantial amount of protein was extracted by boiling water along with a small amount of hyaluronic acid.

SUMMARY

Pig umbilical cord was found to be nearly as rich a source of hyaluronic acid as human umbilical cord. The products derived from pig cord contained approximately the same proportion of nitrogen, acetyl, and glucosamine as human cord products and were also hydrolyzed by the enzyme

hyaluronidase. Hyaluronic acid preparations from pig cord were nitrated and acetylated in the same manner, and inhibited the hyaluronidase-hyaluronic acid system to about the same extent as similar inhibitors made from human cord preparations. In comparing the hyaluronic acid preparations from pig umbilical cord with those from human cord, the essential difference was in the relative viscosity. The pig cord products were appreciably less viscous.

Acetone storage of pig cords prior to processing enhanced the quality of the hyaluronic acid obtained and simplified the extraction and purification procedures, although the introduction of a boiling water treatment into the extraction procedure offered no particular advantage.

BIBLIOGRAPHY

1. Meyer, K., *Physiol. Rev.*, **27**, 335 (1947).
2. Meyer, K., and Palmer, J. W., *J. Biol. Chem.*, **107**, 629 (1934).
3. Meyer, K., and Palmer, J. W., *J. Biol. Chem.*, **114**, 689 (1936).
4. Hadidian, Z., and Pirie, N. W., *Biochem. J.*, **42**, 260 (1948).
5. McClean, D., *Biochem. J.*, **37**, 169 (1943).
6. Sevag, M. G., *Biochem. Z.*, **273**, 419 (1934).
7. Markham, R., *Biochem. J.*, **36**, 790 (1942).
8. Elson, L. A., and Morgan, W. T. J., *Biochem. J.*, **27**, 1824 (1933).
9. Hadidian, Z., and Pirie, N. W., *Biochem. J.*, **42**, 266 (1948).

CHEMISTRY OF THE CHICK EMBRYO

VI. ACCUMULATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE*

By MILTON LEVY AND NELSON F. YOUNG†

(*From the Department of Chemistry, New York University College of Medicine, New York*)

(Received for publication, June 22, 1948)

Despite voluminous literature describing the chemical changes which accompany development of the chick embryo, there are few quantitative data on the occurrence of respiratory and glycolytic enzymes and coenzymes. Since these catalysts are concerned with reactions which may be available for the processes of growth and differentiation, more knowledge of their activities during the incubation period is desirable.

The functional existence, in early chick embryos, of DPN and of a glycolytic system requiring DPN (diphosphopyridine nucleotide, coenzyme I) has been denied by Needham and Lehmann (10). However, Meyerhof and Perdigan (8) were able to demonstrate phosphorylative glycolysis and DPN activity in 4 to 9 day chick embryos. Novikoff, Potter, and LePage (11) extended these observations to the estimation of the amounts of typical intermediates in 3 to 10 day embryos, the demonstration of phosphorylative glycolysis in extracts, and the existence of significant quantities of DPN in extracts. None of the data is sufficiently extensive for the evaluation of the accumulation rate of DPN in the developing chick. The present report presents the results of the estimation of DPN in about 250 chick embryos ranging in age from 2 to 19 days.

EXPERIMENTAL

Test System—The method of Myrbäck (9) was selected as the most suitable for this study. Dried brewers' yeast, after being washed with water, n the presence of hexose diphosphate, glucose, manganese, and phosphate ions produces carbon dioxide only if DPN is also present. The rate of carbon dioxide production is strictly proportional to the DPN concentration over a considerable range. This method is both specific and sensitive nd the rate of reaction is conveniently followed in standard Barcroft-Varburg respirometers. The test system adopted for use in the present ork was as follows: 300 mg. of apozymase (washed yeast preparation); .3 cc. of 10 per cent sodium hexose diphosphate; 0.2 cc. of 20 per cent glucose; 0.1 cc. of 16 per cent $MnCl_2 \cdot 6H_2O$; 1.5 cc. of 0.10 M phosphate, pH

---

* Aided by a grant from the Ella Sachs Plotz Foundation.

† Present address, Sloan-Kettering Institute for Cancer Research, New York.

6.5; 0.9 cc. of DPN solution in 0.1 M phosphate, pH 6.5; gas phase, air; temperature, 30°; fluid volume, 3.0 cc.

The apozymase was prepared from "2040" dried brewers' yeast (Fleischmann) by the method of Kensler, Dexter, and Rhoads (4). Drying was effected by vacuum distillation of the water from the shell-frozen yeast suspension. The dried apozymase was found to be stable for several months if kept dry and cold.

Hexose diphosphate was prepared by the method outlined by Robinson and Morgan (13).

DPN was prepared by the method of Williamson and Green (15). Our preparation was compared in the test system with two samples of DPN obtained from different sources and of different but known purities. The purity of our sample as indicated by these experiments was 80 per cent. Both pentose determinations (7) and phosphorus determinations (1) were consistent with 81 per cent purity. Allowance was made for 80 per cent purity in the calculations.

Under the conditions of our experiments the extra carbon dioxide produced per hour was proportional to the DPN added in the range of 0 to 15 γ. All determinations were done in this range. Our apozymase and DPN gave 65 per cent of the carbon dioxide production rate observed by Meyerhof and Perdigan (8) per microgram of DPN. It remained unchanged throughout the experimental period.

Embryological Material—Fertile eggs, from Rhode Island red hens, collected in trap nests and received in 2 dozen lots within 48 hours of laying were incubated in a commercial incubator at 38.5° and 50 to 60 per cent relative humidity. The eggs were rotated twice daily and removed for analysis in groups of four.

Preparation of Tissues—The grinding of animal tissues liberates a heat-labile system which rapidly destroys DPN. In minces of chick embryo tissues this inactivation (8) proceeds rapidly even at temperatures just above freezing. Since the enzyme system responsible for this inactivation is destroyed by heating at 80° (3), the following procedure for reducing the embryos to a homogeneous suspension was adopted. The embryos were removed from the egg and freed from extraembryonic membranes. After superficial drying and accurate weighing, the smaller embryos (3 to 1000 mg.) were dropped whole into 20 volumes of 0.1 M phosphate buffer, pH 6.5, and heated for 5 minutes at 80°. They were then cooled rapidly in a cold water bath and ground in a glass homogenizer as described by Potter and Elvehjem (12). The large embryos were dropped into 20 volumes of buffer previously heated to 80–90° after their weights had been obtained. They were then cut into small pieces with scissors while in the hot solution. This mixture was maintained at 80° for 5 minutes. After rapid cooling it was homogenized as described above or in a Waring blendor if the bones

Table I

Recovery Experiments and CO_2 Production with Varying Amounts of Tissue

DPN added, γ	0	7.5	0	5*	7.5*	0
Tissue added, *mg*	0	0	34	34	34	102
CO_2 production per hr., *c.mm.*	137	424	377	567	666	857
Net CO_2 production per hr., *c.mm.*		287	240	430	523	720
DPN found, γ			6.3	11.2	13.7	18.8
Calculated, γ				11.3	13.8	18.9
Recovery, %				99	99	99

* Incubated 0.5 hour with heat-inactivated tissue.

Table II

Accumulation of DPN in Chick Embryos

No. of embryos in group	Average weight	Average age, calculated	Average DPN
	mg.	*days*	*γ per gm. wet weight*
14	3.06	2.06	130
12	11.4	2.62	140
8	18.9	2.86	133
8	28.5	3.10	144
7	74.5	3.65	109
12	122.0	4.06	111
10	204.3	4.86	105
10	292	5.37	122
10	548	6.40	133
10	680	6.78	140
10	886	7.29	148
10	1,041	7.64	162
9	1,229	8.00	151
10	1,386	8.26	144
10	1,803	8.89	132
10	2,200	9.40	121
10	2,896	10.14	137
10	3,526	10.72	142
10	4,514	11.46	148
10	6,409	12.65	158
10	9,092	13.93	171
10	11,350	14.79	177
10	12,970	15.35	192
10	15,580	16.18	166
10	21,200	17.58	155
10	27,100	18.84	125

were so large as to interfere with adequate grinding in the glass homogenizers. For analysis 0.9 cc. portions of these 4.8 per cent suspensions were pipetted into the Warburg vessels for DPN estimation.

Results

Table I demonstrates the almost complete recovery of DPN added to 10 day-old embryo tissue (heated as above) and incubated with it for half an hour at 37°.

It will be noted that the control vessel to which no DPN was added also produced some carbon dioxide. This was apparently due to residual DPN or other factors in the yeast and was subtracted as a blank from each determination. With each set of four determinations, the blank carbon dioxide production and the CO_2 per hour per microgram of DPN were determined.

Determinations of DPN were made on a total of 252 embryos. In all but the very early ages the estimations were made on individuals. The embryos were arranged in a series according to weight and averaged in groups of about ten, both as to the DPN content and to the age as calculated from the average weight. The rationale of using a calculated age is discussed by Levy and Palmer (5).

Table II summarizes this data showing the relationship between DPN concentration and calculated age.

DISCUSSION

Needham and Lehmann (10) reported that they were unable to demonstrate the presence of DPN in embryonic chick tissue. On the basis of this finding and from other considerations, they proposed that glycolysis in chick embryo tissue does not proceed by way of the usual glycolytic system as found in adult mammalian tissue. From the present study, it is apparent that although DPN is present in low concentration as compared to mammalian tissue, it is by no means absent. Our values are in the range reported by Meyerhof and Perdigan (8) (1.5 to 3.0 mg. per gm. of dry weight, equivalent to 120 to 240 γ per gm. of wet weight) and 3 to 6 times those reported by Novikoff, Potter, and LePage (11) (4 to 6 γ per 100 gm., equivalent to about 35 γ per gm. of wet weight). The agreement between the data of Meyerhof and Perdigan (8) and ours is not surprising, since the methods used were not significantly different. On the other hand, Novikoff, Potter, and LePage (11) used malic dehydrogenase as the test system. However, the system was also calibrated with DPN and since both methods are comparative this could not account for the 3- to 6-fold difference in the values found. It seems most likely that the preparation of the samples for analysis is accountable. The lower values were obtained by a method which depends on freezing and cold to prevent enzymatic destruction of DPN. It seems probable that in the preparation or test period, or both, some DPN was destroyed by the surviving enzyme in the tissue extracts.

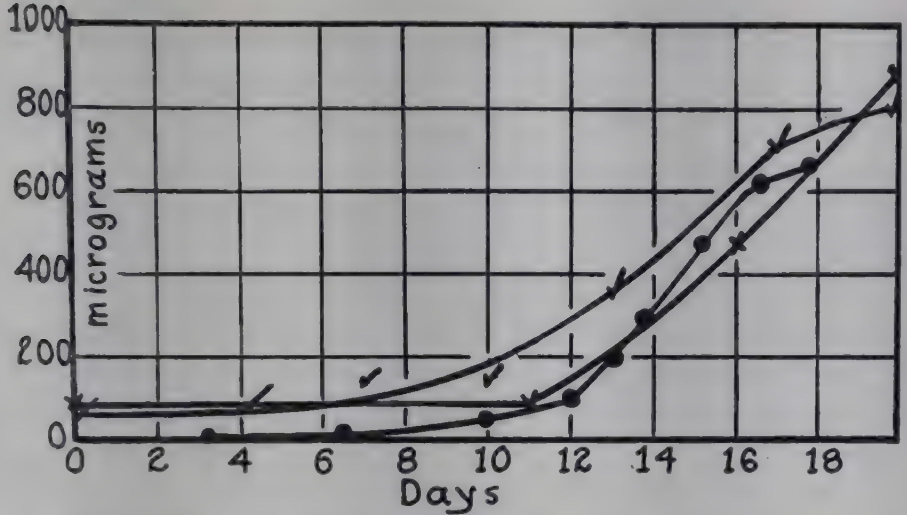


FIG. 1. Nicotinic acid per egg and DPN per embryo during growth. ✓ nicotinic acid per egg (14), × nicotinic acid per egg (2), ● nicotinic acid equivalent to the DPN per embryo in Table II.

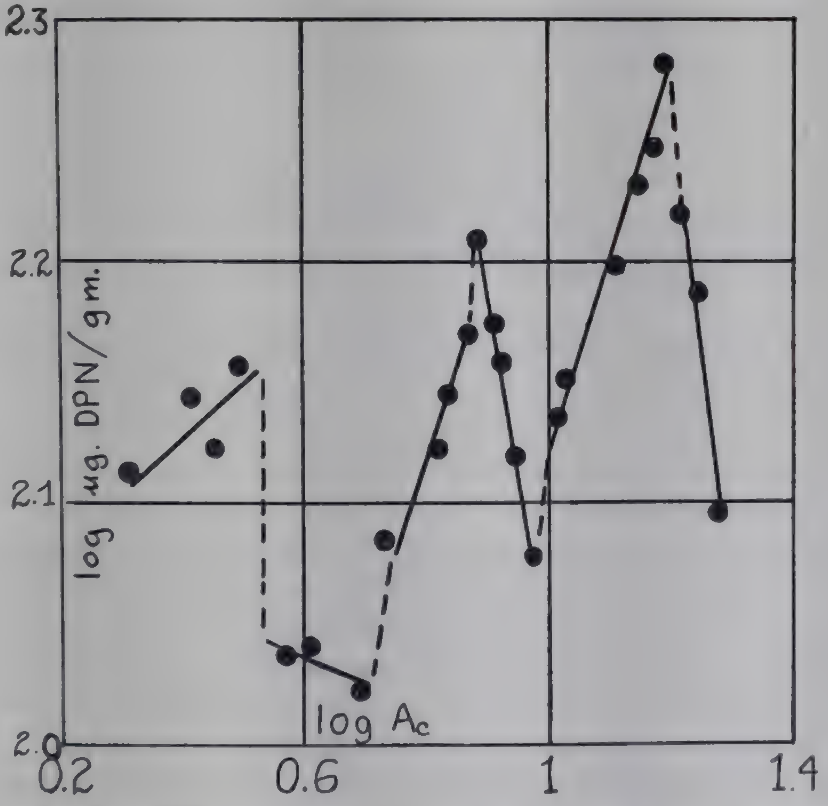


FIG. 2. Relative accumulation diagram of chick embryo DPN. A_c is the age in days calculated from the weight. Each straight line segment (phase) conforms to the equation $\log Q/W = (i_Q - i_W) + (a_Q - a_W) \log A_c$. The dashed portions are at interphases and are less certain. The values of i_Q and a_Q for DPN are given in Table III as the phase parameters.

It is of interest to compare the amount of nicotinic acid bound as DPN in the embryo with the quantity of nicotinic acid in the whole egg contents. The data of Handler and Dann (2) and of Snell and Quarles (14) both indicate an active synthesis of nicotinic acid by the embryo. Fig. 1 shows the total nicotinic acid in the egg as determined by these workers. Also shown is the nicotinic acid equivalent to the DPN found in the present study. Although the breed of chick is different in each curve, the comparison shows that in the later stages of development a large part of the total nicotinic acid is to be found in the DPN. The data suggest that the amount of nicotinic acid synthesized by the chick is limited chiefly to the requirement for the formation of this coenzyme. Since this substance is part of the functional structure of the chick (rather than a stored material), it seems unlikely that the availability of more nicotinic acid or its precursors would have any effect on the amount of DPN formed during development. We therefore cannot conclude that the amount of DPN formed is limited by the chick's ability to synthesize nicotinic acid.

TABLE III

Phase Parameters for Accumulation of DPN in Chick Embryo

Log micrograms of DPN = $i_Q + a_Q$ log age in days. See Fig. 2.

Interphases, *days*	(2.0)*		3.5		4.5		7.6		9.6		16		(19)*
a_Q		5.29		5.21		4.34		1.78		4.36		1.70	
i_Q		−2.02		−2.08		−1.58		0.62		−1.64		0.37	

* The figures in parentheses indicate the beginning or end of the period of data.

Fig. 2 is a "relative accumulation diagram" (5) on which the logarithms of DPN concentrations in micrograms per gm. of wet weight of embryo are plotted against the logarithms of the corresponding ages in days. We have found such plots useful in demonstrating the periods of apparent regularity of growth implicit in a linear relationship between the ordinates of logarithms of amount of material (in this case DPN) and logarithms of age. These periods of regularity (phases) are terminated at times (interphase times) when an abrupt adjustment of constants is necessary to describe the data. Six phases separated by five interphases are evident in Fig. 2. From the slopes and intercepts of the phase lines and the appropriate weight-age relationships (5) the "phase" parameters of Table III were obtained. It is noted that the interphase at 4.5 days has been found in all the accumulation data so far obtained, that 7.6 days is an interphase in cytochrome oxidase accumulation (7.9 days) (6), that 3.5 and 9.6 days are interphases in dipeptidase accumulation (3.6 and 9.6 days), and that 16 days is an interphase in nitrogen accumulation (5). The interpretation of these coincidences remains for the future.

SUMMARY

1. The diphosphopyridine nucleotide contents of chick embryos have been estimated from 2 to 19 days incubation.

2. The data indicate that the coenzyme is present in the earliest embryos and contains most of the nicotinic acid of the egg in older embryos.

3. The accumulation of the coenzyme follows the pattern of periods of constant multiplication rate in logarithmic time units previously shown to hold for other materials.

BIBLIOGRAPHY

1. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
2. Handler, P., and Dann, W. J., *J. Biol. Chem.*, **140**, 739 (1941).
3. Handler, P., and Klein, J. R., *J. Biol. Chem.*, **143**, 49 (1942).
4. Kensler, C. J., Dexter, S. O., and Rhoads, C. P., *Cancer Res.*, **2**, 1 (1942).
5. Levy, M., and Palmer, A. H., *J. Biol. Chem.*, **150**, 271 (1943).
6. Levy, M., and Young, N. F., *J. Biol. Chem.*, **175**, 73 (1948).
7. Mejbaum, W., *Z. physiol. Chem.*, **258**, 117 (1939).
8. Meyerhof, O., and Perdigan, E., *Enzymologia*, **8**, 353 (1940).
9. Myrbäck, K., *Ergeb. Enzymforsch.*, **2**, 139 (1933).
10. Needham, J., and Lehmann, H., *Biochem. J.*, **31**, 1210 (1937).
11. Novikoff, A. B., Potter, V. R., and LePage, G. A., *J. Biol. Chem.*, **173**, 239 (1948).
12. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
13. Robinson, R., and Morgan, W. T. J., *Biochem. J.*, **24**, 119 (1930).
14. Snell, E. E., and Quarles, E., *J. Nutr.*, **22**, 483 (1944).
15. Williamson, S., and Green, D. E., *J. Biol. Chem.*, **135**, 345 (1940).

THE MEASUREMENT OF TURNOVER OF THE VARIOUS PHOSPHOLIPIDES IN LIVER AND PLASMA OF THE DOG AND ITS APPLICATION TO THE MECHANISM OF ACTION OF CHOLINE

BY D. B. ZILVERSMIT,* C. ENTENMAN, AND I. L. CHAIKOFF

(From the Division of Physiology, University of California Medical School, Berkeley)

(Received for publication, May 3, 1948)

Experimental evidence presented in earlier reports failed to support the thesis that phospholipides are instrumental in the transport of fatty acids between organs (1). Another opportunity for examining this question presented itself in connection with the lipotropic action of choline, which has been attributed to a stimulation in the metabolism of phospholipides such as to increase the transport of fatty acids out of the liver (2).

The observations of Entenman *et al.* on dogs injected with P^{32} demonstrated that choline increases the specific activity of the choline-containing phospholipide P of plasma and of liver (3). Such increases in liver and plasma could either result from an increase in the turnover of the phospholipides *per se* or merely reflect an increase in the specific activity of their precursors unassociated with an actual increase in turnover of these phospholipides. To differentiate between these two possibilities, it became necessary to calculate the turnover rates of liver and plasma phospholipides. This has been attempted in the present investigation.

To calculate the turnover of a compound, one has to know the specific activity-time relations of that compound and of its immediate precursor (4). This measurement can be made for plasma lecithin and for plasma sphingomyelin because it has been shown by Fishler *et al.* that the immediate precursors of these plasma phospholipides are their corresponding liver phospholipides (5).

So far as the liver phospholipides are concerned, however, their precursors have not been established with certainty, and hence the determination of the turnover rates of liver phospholipides is rendered difficult if not impossible.

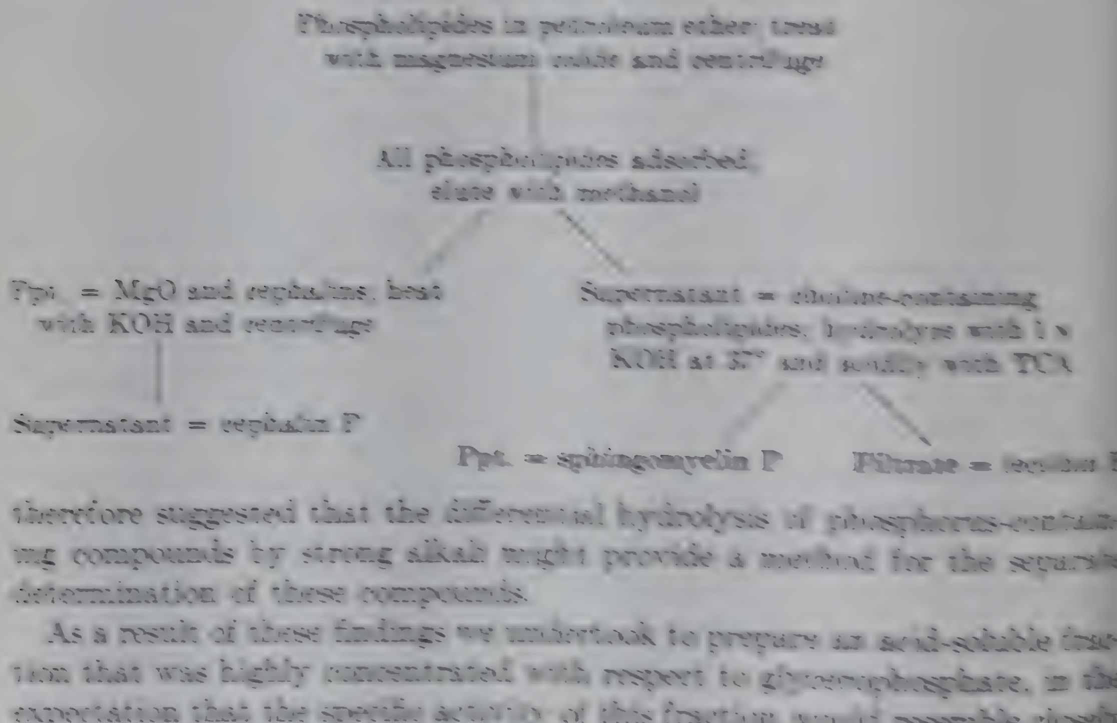
Several investigators (6–8) have attempted to identify the phosphorus-containing precursors of liver phospholipides. Thus Flock and Bollman have shown that after the injection of P^{32} into birds the specific activity of liver glycerophosphate P is higher than that of liver phospholipide P, and from this observation it is conceivable that the former is the precursor of lecithin and cephalin in the liver (7). It therefore became of interest to us to determine whether in the dog glycerophosphate satisfies more specific requirements proposed earlier for identification of a precursor (4).

---

* Fellow of the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

The separation of glycerophosphate from other acid-soluble P compounds by means of its barium salt is a laborious procedure and is open to criticism. Kursanov (9), for example, observed that, at the pH at which inorganic phosphate is completely precipitated by barium, most of the glycerophosphate is also removed from solution. He also found that after the heating of glycerophosphate with 10 per cent NaOH for 3 hours or more only 20 per cent of the phosphorus was split off, whereas the same treatment caused the loss of 80 per cent of the phosphate of hexose monophosphate. He therefore suggested that the differential hydrolysis of phosphorus-containing compounds by strong alkali might provide a method for the separate determination of these compounds.

DIAGRAM I


Phospholipides in petroleum ether; treat with magnesium oxide and centrifuge
All phospholipides adsorbed; elute with methanol
Ppt. = MgO and cephalins; heat with KOH and centrifuge
Supernatant = choline-containing phospholipides; hydrolyze with 1 N KOH at 37° and acidify with TCA
Supernatant = cephalin P
Ppt. = sphingomyelin P
Filtrate = lecithin P

As a result of these findings we undertook to prepare an acid-soluble fraction that was highly concentrated with respect to glycerophosphate, in the expectation that the specific activity of this fraction would resemble closely the specific activity of glycerophosphate.

EXPERIMENTAL

Methods

Separation of Phospholipides into Lecithins, Cephalins, and Sphingomyelins (Diagram I)—The phospholipides of liver and plasma were first extracted with alcohol and ether and finally dissolved in petroleum ether by a procedure described elsewhere (10). Choline-containing and non-choline-containing phospholipides were then separated after the manner of Taurog *et al.* (11). In this procedure the choline-containing phospholipides were

luted with methanol, whereas the cephalin fraction remained with the
agnesium oxide. In the case of plasma phospholipides such a separation
as not necessary, since it was shown by Taurog *et al.* that the phospho-
pides of dog plasma consist almost entirely of lecithins and sphingomye-
ns (12).

The choline-containing phospholipides were next suspended in 1 N KOH
nd incubated at 37° for 24 hours, so as to separate lecithin and sphingo-
yelin P by the method of Schmidt *et al.* (13). An aliquot of the trichloro-
cetic acid filtrate (TCA, Diagram 1), obtained by the method of Schmidt
al., was used for the determination of lecithin P^{32}. Another aliquot of
is filtrate was oxidized with perchloric acid and its P^{31} content determined
olorimetrically (14).

Immediately after the filtration that separated lecithin and sphingo-
yelin phosphorus (Diagram 1), the precipitate was washed with 10 per
ent trichloroacetic acid and was then removed from the filter paper with
few cc. of water. The precipitate was next dissolved by adding to it 1
c. of 10 per cent NaOH and heating to 50-60°. An aliquot was then taken
r the measurement of its P^{32} content and the rest of the solution ashed
ith perchloric acid for the determination of P^{31}.

To determine the specific activity of the cephalin P, 1 to 2 gm. of the
IgO (which contained the cephalin phosphorus) were heated overnight on
steam bath with 25 cc. of 1 N KOH. After centrifugation the super-
atant was transferred to another vessel and the MgO washed once with
0 cc. of distilled water. More than 90 per cent of the P^{32} adsorbed on the
IgO was recovered in the KOH phase. The P^{32} and P^{31} content of the
OH solution was determined as described above for lecithin.

Test for Separation of Sphingomyelin P from Lecithin P—Schmidt *et al.*
ested their method for the selective saponification of phospholipides on
nown mixtures of pure lecithin, cephalin, and sphingomyelin (13). For
ur purpose it seemed advisable to investigate the separation of lecithin
nd sphingomyelin phosphorus in dog plasma, a fluid that contains only
hese two phospholipides.

A sample of dog plasma was divided in two portions. One was treated
the usual way with alcohol and ether to remove its phospholipides. The
xtracted phospholipides were then subjected to hydrolysis at 37° and
phingomyelin determined as the difference between total P and lecithin P.

The other portion of the plasma was treated with colloidal iron according
the method of Folch and Van Slyke (15). In this way an extract of
lasma phospholipides was prepared free of non-lipide nitrogen. The N:P
tio of this extract was then used to calculate the sphingomyelin content
f the phospholipides. The results of the two procedures are compared
Table I.

It should be noted here that the procedure of Schmidt *et al.* is based on the determination of a small difference between the total phospholipide P and the lecithin P; hence the percentage error in the values for sphingomyelin P obtained by this method may be much larger than the errors in the individual P determinations. The determination of the specific activity of sphingomyelin is, however, not subject to this error, since the ratio of $P^{32}:P^{31}$ determined in the same solution is not affected by losses of P during transfers or washings.

Separation of Acid-Soluble Phosphorus Fraction into Alkali-Stable and Alkali-Hydrolyzable Portions—About 10 gm. of ground liver were homogenized with 10 cc. of 10 per cent trichloroacetic acid (TCA, Diagram 2) and the mixture centrifuged. The supernatant was made up to a volume of 50 cc. The trichloroacetic acid extract was next neutralized with 16 N NaOH. 5 gm. of NaOH (pellets) were then added to each tube and the mixture heated in a boiling water bath for 3 hours. After it had cooled, the hydrolysate was neutralized with concentrated HCl. 10 cc. of a magnesia mixture[1] and 7 cc. of 28 per cent aqueous NH_3 were then added in this order. The magnesium ammonium phosphate which formed was allowed to settle overnight. After filtration and thorough washing with 3 per cent NH_3, the phosphate was dissolved in 1.2 N HCl. Filtrates A and B (Diagram 2) were used for the determination of the specific activity of the alkali-stable and of the alkali-hydrolyzable P, respectively.

TABLE I

Comparison of Sphingomyclin Content of Plasma by N:P Ratios and by Method of Schmidt et Al.

Sample No.	Sphingomyelin as per cent of total phospholipides	
	From N:P	Method of Schmidt *et al.*
1	18	21*
2	28	20†
3	12	14†

* Average of two determinations.
† Average of three determinations.

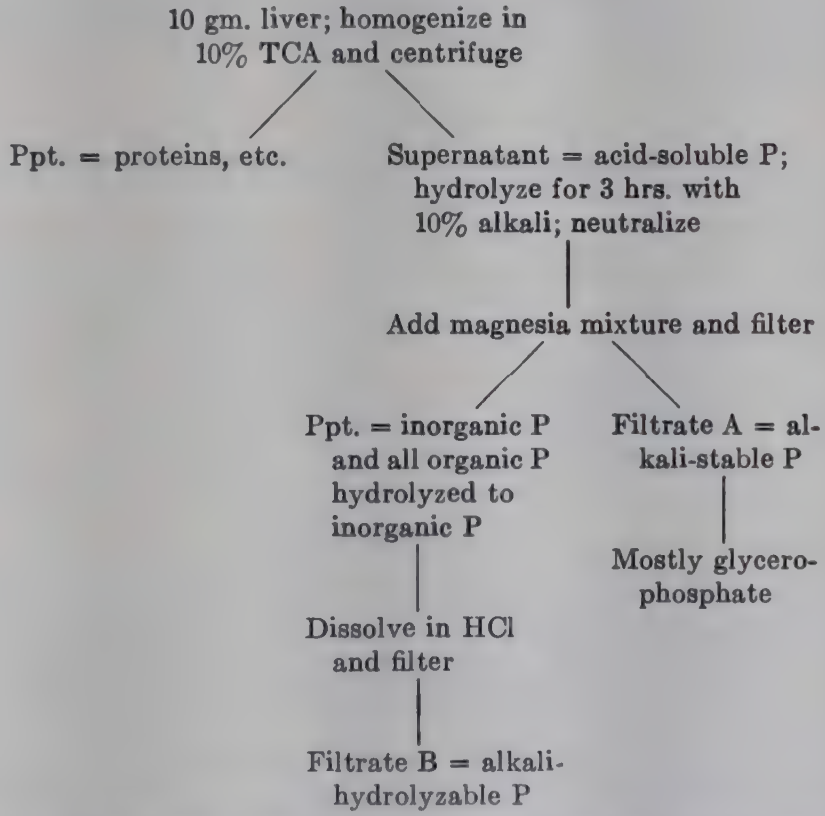
---

[1] The magnesia mixture consisted of 55 gm. of $MgCl_2 \cdot 6H_2O$, 70 gm. of NH_4Cl, 650 cc. of H_2O, and 350 cc. of 10 per cent aqueous NH_3. Kurssanov (9) showed that in the precipitation with the magnesia mixture the final concentration of ammonia is very important. Insufficient concentrations of ammonia were shown to result in incomplete precipitation of the inorganic phosphate, whereas high doses of ammonia brought about the precipitation of glycerophosphate. In our experiments we used the optimal aqueous NH_3 concentration proposed by Kurssanov for the separation of inorganic phosphate from glycerophosphate.

Test for Exchange Reactions during Fractionation Procedure—Since the glycerophosphate was heated in the presence of strong alkali, it seemed possible that the phosphorus moiety might exchange with the phosphate of the alkali-hydrolyzable fraction. Chargaff (16) has already presented data to show that such an exchange reaction does *not* occur in an acid medium.

In order to test whether exchange took place under the conditions of the procedure described above, glycerophosphate containing 4 mg. of P was heated for 5.5 hours on the steam bath with 4 mg. of radioactive phosphate in a 10 per cent NaOH medium. After neutralization and addition of the magnesia, the sample was kept cold overnight in order to remove all inorganic phosphate. Almost all the P of the filtrate was found to consist of glycerophosphate as measured by Burmaster's method (17). Only 0.03 per cent of the P^{32} was found in this fraction. This means that no exchange had taken place under conditions of our analytical procedure.

DIAGRAM 2


10 gm. liver; homogenize in 10% TCA and centrifuge
Ppt. = proteins, etc.
Supernatant = acid-soluble P; hydrolyze for 3 hrs. with 10% alkali; neutralize
Add magnesia mixture and filter
Ppt. = inorganic P and all organic P hydrolyzed to inorganic P
Filtrate A = alkali-stable P
Mostly glycerophosphate
Dissolve in HCl and filter
Filtrate B = alkali-hydrolyzable P

Preparation of Radioactive Glycerophosphate—250 gm. of liver were obtained from a dog which had received intravenously radioactive phosphate 16 hours previously. Its phospholipides were first isolated as described elsewhere (10) and then redissolved in petroleum ether and reprecipitated with acetone.

400 cc. of barium hydroxide containing 25 gm. of $Ba(OH)_2 \cdot 8H_2O$ were added to 4 gm. of phospholipides which had been dissolved in 200 cc. of hot methanol; thorough shaking was employed during the mixing to insure emulsification of the phospholipide. Most of the methanol was evaporated by heating the mixture for 2 hours on the steam bath. About 4 cc. of concentrated H_2SO_4 were next added to make the solution acid as indicated by brom-cresol green and the precipitate which formed filtered off. The filtrate was shaken three times with equal volumes of petroleum ether containing 5 per cent chloroform to remove unhydrolyzed phospholipides and other fatty materials. The petroleum ether washings contained practically no radioactivity. According to Burmaster's method of measurement (17), practically all of the phosphorus in the water phase was present as glycerophosphate.

Table II

Specific Activities of Phospholipides and Acid-Soluble Fractions in Two Different Lobes of Liver

Dog No.	Fraction	Lobe A	Lobe B
22	Lecithin	11.1	11.2
	Cephalin	8.74	8.70
	Sphingomyelin	5.97	6.09
6	Alkali-stable	14.2	14.7
	Alkali-hydrolyzable	14.8	14.9

Animal Preparations

Normal and fat-fed dogs were used in this investigation. The former were fed a high protein (lean meat) diet for several weeks before the experiment. The latter received a high fat, low protein diet (its composition has been recorded elsewhere (3)) for 4 days before being used. All dogs were first fasted for 24 hours and then lightly anesthetized with nembutal and kept so for the duration of the experiment. Several samples of liver were excised as described in a previous report (3). Each sample was ground thoroughly; one aliquot was transferred to 95 per cent alcohol for determination of its phospholipide content, whereas another sample was homogenized in 10 per cent trichloroacetic acid for the determination of the two acid-soluble P fractions (Diagram 2).

Since samples of liver were taken from various lobes, it became necessary to inquire into the degree of variation of all P fractions found at a single time in the lobes of the liver. This has already been done for two phospholipide fractions (3). The results presented in Table II for two separate lobes lend further support to the view that the specific activity of a phosphorus compound measured in a small sample of liver is a reliable index of the specific activity of that compound in the whole liver.

Results

Normal Dogs—Biopsy samples of the liver (10 to 15 gm.) were excised from Dog A at intervals of 3, 5, 9, and 12 hours, and from Dog B at 12.5, 16.5, and 21 hours after the administration of P^{32}. The samples were analyzed for total phospholipide P, alkali-stable P, and alkali-hydrolyzable acid-soluble P. The values for the specific activities of these three fractions, expressed as percentages of the injected P^{32} per mg. of phospholipide P, are plotted in Fig. 1.

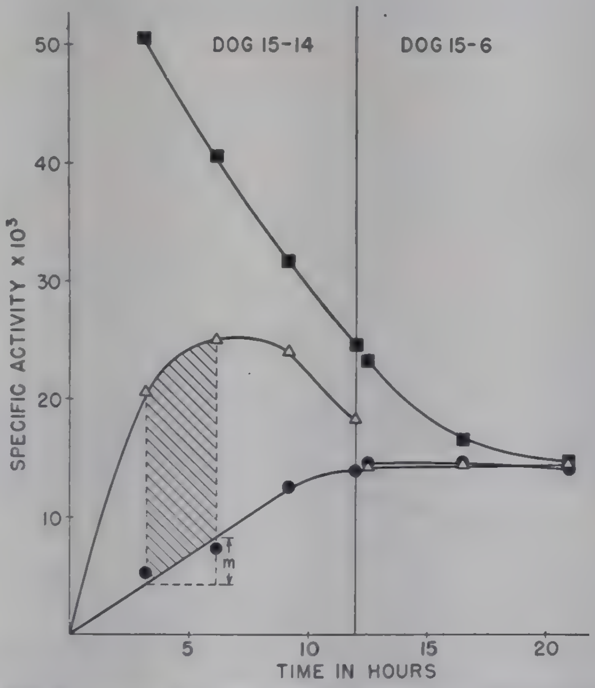


FIG. 1. Specific activity-time relation of liver alkali-hydrolyzable P (■), liver alkali-stable P (△), and liver phospholipide P (●).

It was pointed out by Zilversmit *et al.* (4) that the following relations exist between a compound and its precursor: (1) during the early intervals after a single injection of P^{32}, the specific activity of the precursor is higher than that of the compound; (2) at the time when the specific activity of the compound is maximal, it is equal to that of the precursor; (3) thereafter the specific activity of the precursor is lower than that of the compound. These criteria for a precursor hold only for single compounds. Although, as judged by these criteria, both alkali-stable and alkali-hydrolyzable phosphorus could be considered precursors of liver phospholipide phosphorus, the latter should be dismissed from consideration because its phosphorus is derived from several different phosphorus-containing compounds. The alkali-stable fraction, however, consists largely of glycerophosphate, and our main interest was to test whether this compound satisfies the precursor requirements.

It should next be pointed out that the fraction designated phospholipide consists of several compounds. We therefore proceeded to investigate the specific activity-time relations between each of the acid-soluble fractions and individual types of phospholipides; namely, lecithins, cephalins, and sphingomyelins. The results obtained on three dogs (Nos. 22, 36, and 56) are shown graphically in Figs. 2 to 4. Dogs 22 and 36 were fed the lean meat diet, whereas Dog 56 received the high fat diet.

For all three dogs, the specific activities[2] of the lecithin fraction were higher than those of the other phospholipides. Figs. 2 to 4 show that the alkali-stable fraction meets all the requirements set forth above for a lecithin precursor.

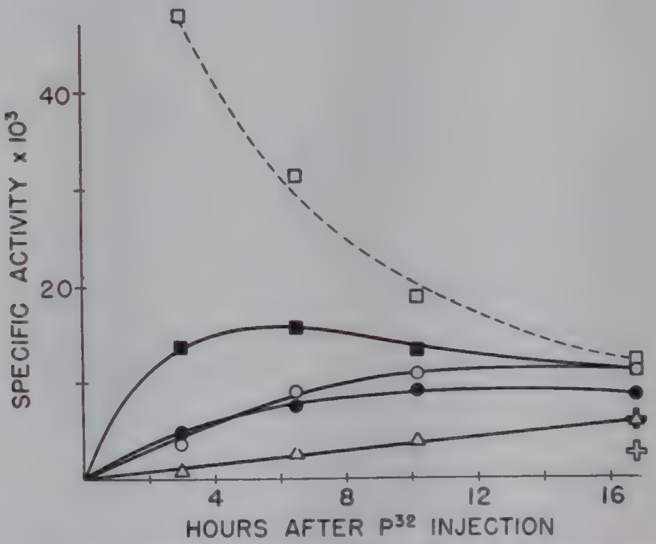


FIG. 2. Specific activity-time relations (Dog 22) of liver alkali-hydrolyzable P (□), liver alkali-stable P (■), liver lecithin P (○), liver cephalin P (●), and liver sphingomyelin P (△), and the specific activities of plasma lecithin P (✚) and plasma sphingomyelin P (open symbol).

Further evidence as to whether alkali-stable phosphorus is a precursor of liver lecithin phosphorus was sought in choline-treated dogs in which the shape of the liver lecithin specific activity curve is radically changed. Maintenance of the relations outlined above between lecithin phosphorus and alkali-stable phosphorus under the new conditions provided by choline treatment would, of course, add support to the possibility that the alkali-stable phosphorus is the sought for precursor.

Fat-Fed, Choline-Treated Dogs—Three dogs were fed high fat diets for 4 days and then fasted for 24 hours before the administration of 300 mg. of choline per kilo of body weight. The P^{32} was injected intravenously 30 minutes later. The results on these three dogs are shown in Figs. 5 to 7.

---

[2] All specific activities are expressed as percentages of the injected P^{32} per mg. of total P.

A pronounced increase in the specific activities of alkali-hydrolyzable P, alkali-stable P, and lecithin P was observed in two of the three dogs. Thus the maximum specific activity of the liver lecithin in the choline-treated dog was twice that of the untreated dogs. Despite the change in shape of the specific activity-time curve for liver lecithin that resulted from the administration of choline, the curve for alkali-stable phosphorus retained features sufficient to characterize it as a possible precursor of liver lecithin.

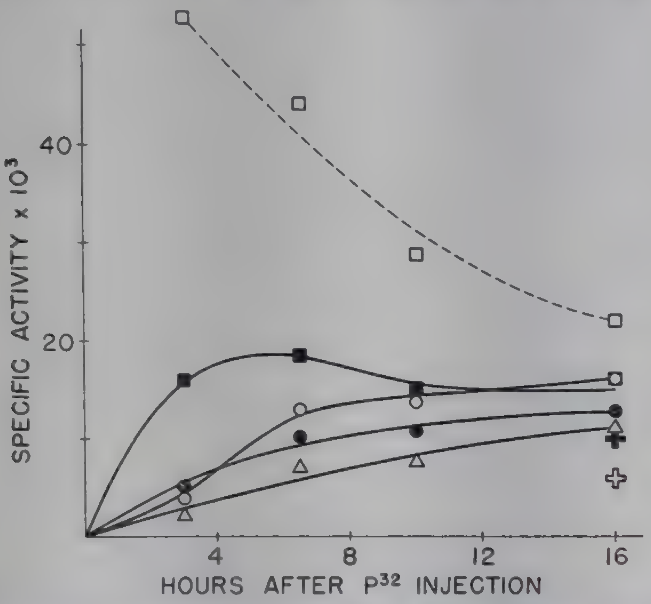


FIG. 3. Specific activity-time relations (Dog 36) of liver alkali-hydrolyzable P (□), liver alkali-stable P (■), liver lecithin P (○), liver cephalin P (●), and liver sphingomyelin P (△), and the specific activities of plasma lecithin P (✚) and plasma sphingomyelin P (open symbol).

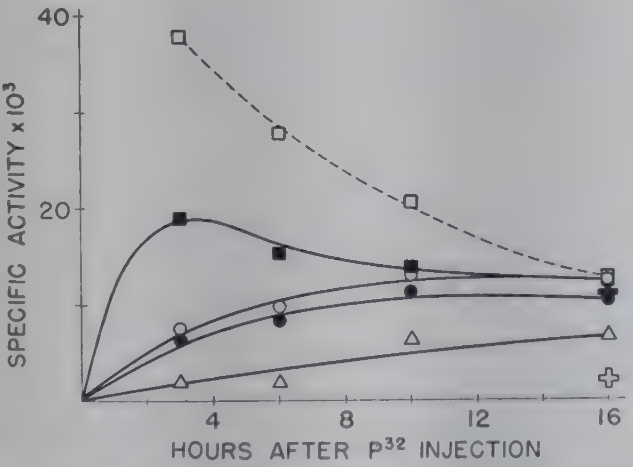


FIG. 4. Specific activity-time relations (Dog 56) of liver alkali-hydrolyzable P (□), liver alkali-stable P (■), liver lecithin P (○), liver cephalin P (●), and liver sphingomyelin P (△), and the specific activities of plasma lecithin P (✚) and plasma sphingomyelin P (open symbol).

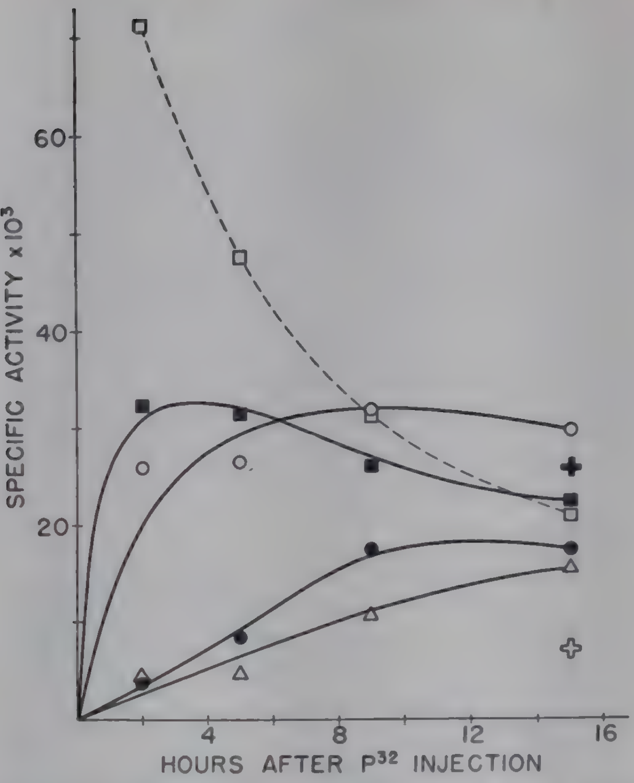


FIG. 5. Specific activity-time relations (Dog 44) of liver alkali-hydrolyzable P (□), liver alkali-stable P (■), liver lecithin P (○), liver cephalin P (●), and liver sphingomyelin P (△), and the specific activities of plasma lecithin P (✚) and plasma sphingomyelin P (open symbol).

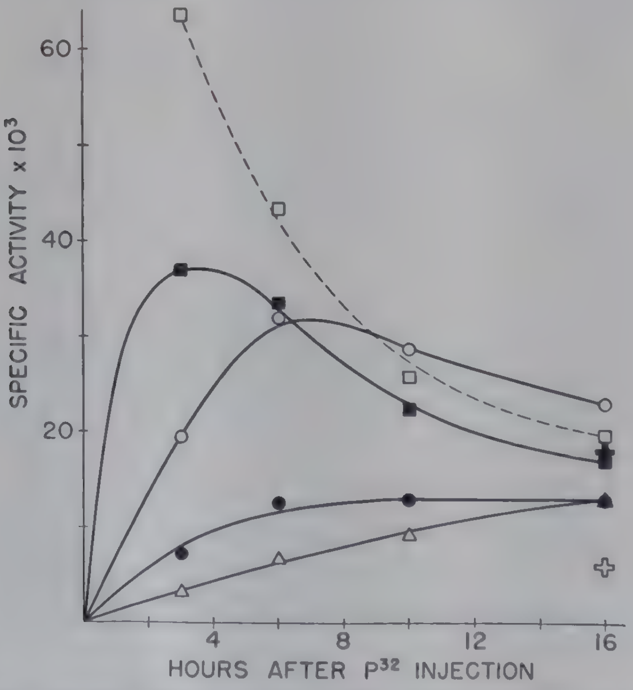


FIG. 6. Specific activity-time relations (Dog 45) of liver alkali-hydrolyzable P (□), liver alkali-stable P (■), liver lecithin P (○), liver cephalin P (●), and liver sphingomyelin P (△), and the specific activities of plasma lecithin P (✚) and plasma sphingomyelin P (open symbol).

Calculation of Turnover Time of Liver Lecithin

From theoretical considerations presented elsewhere (4) it was concluded that a measure of the turnover time[3] of a phospholipide can be obtained from the specific activity-time curves of the phospholipide and its precursor. The phospholipide turnover time, t_t, is obtained from the ratio of (1) the area bounded by these two curves between two time coordinates to (2) the increase in the phospholipide specific activity in that same time interval. This procedure is illustrated in Fig. 1. Thus,

$$t_t = \frac{\text{shaded area}}{m}$$

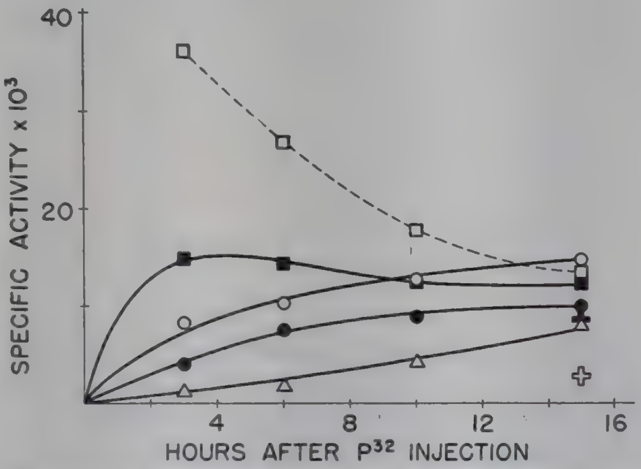


FIG. 7. Specific activity-time relations (Dog 57) of liver alkali-hydrolyzable P (□), liver alkali-stable P (■), liver lecithin P (○), liver cephalin P (●), and liver sphingomyelin P (△), and the specific activities of plasma lecithin P (✚) and plasma sphingomyelin P (open symbol).

For the measurement of t_t, it is necessary to use the specific activity at an early interval because (1) it is the time when the error in the determination of the difference between the specific activities observed at the beginning and end of the period could be expected to be minimal, since at the early intervals the specific activity of liver lecithin increases most rapidly, and (2) if deleterious effects of surgery occurred, they would be expected to be minimal at the early intervals. Since we have no data on the shape of the curves up to the point when the first liver sample was taken, the calculation had to be applied to the area beyond this point. We therefore chose the time interval between the taking of the first and second liver samples as most reliable for the calculation of t_t.

t_t values for liver lecithin are recorded in Table III. For reasons dealt with above and in "Discussion" below, those based on the alkali-stable fraction as precursor are more reliable.

---

[3] The turnover time of a liver phospholipide is the time required for the turnover of an amount of liver phospholipide equal to that present in the liver.

The results in Table III clearly show that, regardless of which fraction is taken for the calculation, the t_t values for liver lecithin are decreased in the two dogs that responded to choline treatment. Since the total lecithin in the livers of the choline-treated dogs remained normal, this means that liver lecithin is synthesized at a more rapid rate in the choline-treated than in the normal dog.

TABLE III

Turnover Time of Liver Lecithin*

	Dog No.	Weight	Based on alkali-stable specific activity	Based on alkali-hydrolyzable specific activity
		kg.	*hrs.*	*hrs.*
Normal	22	12	5.9	21
	36	13	3.6	15
	56	11	6.1	18
Choline-treated	44	11	1.8	8.7
	45	13	2.0	5.9
	57†	14	5.6	21

* All values are obtained from the areas and specific activities between the first and second points on the curve (*i.e.*, 3 to 6 hours).

† This dog failed to show an increase in the specific activities of liver or plasma phospholipides after the administration of choline. 85 per cent of the dogs treated with choline showed marked increases in phospholipide specific activities. Irregularities in the response of animals towards the lipotropic action of choline are known to occur.

Calculation of Turnover Time for Plasma Lecithin and Sphingomyelin

The specific activities of these two plasma phospholipides were elevated in the choline-treated dog. Since the liver is the source of plasma phospholipides, this means either (1) that the turnover of plasma phospholipides had actually increased or (2) that the change in specific activities merely reflects similar changes in liver phospholipides unassociated with an actual increase in turnover of plasma phospholipides.

In order to obtain the turnover time of plasma sphingomyelin we can make use of the fact that in nearly all cases the specific activity of liver sphingomyelin increases linearly with time. Under such conditions the following relation has been shown to hold (4):

$$\frac{x}{r} = bt_t\left(\frac{t}{t_t} - 1 + e^{-t/t_t}\right) \qquad (1)$$

in which x/r is the specific activity of plasma sphingomyelin at time t, b is the increase in specific activity of liver sphingomyelin per unit of time, and t_t is the turnover time for plasma sphingomyelin.

If the specific activities of plasma sphingomyelin and of liver sphingomyelin at any one time are known (in our experiment we knew the values at the end of the experiment), then the turnover time of plasma sphingomyelin can be calculated from equation (1). The values so obtained are presented in Table IV.

A calculation for turnover time of plasma lecithin is more complex, since the relation observed between the specific activity of liver lecithin and time is not simple. In a previous communication the following equation was derived for the relation between the specific activity of phospholipide phosphorus and that of its precursor when the specific activity of the latter is kept constant (4):

TABLE IV

Turnover Time of Plasma Lecithin and Sphingomyelin*

	Dog No.	Lecithin	Sphingomyelin
		hrs.	*hrs.*
Normal	22	11	12
	36	6	10
	56	10	13
Choline-treated	44	6	11
	45	12	10
	57		21

* For calculation see the text.

$$\frac{x}{r} = a(1 - e^{-t/t_t}) \qquad (2)$$

where x/r is the specific activity of the phospholipide at time t, a the constant specific activity of its precursor, and t_t the turnover time of the phospholipide.

In our experiment the specific activity of the phospholipide precursor did not remain constant (see Figs. 1 to 7). A good approximation of t_t can be obtained when the average specific activity of liver lecithin (in this case the precursor) is substituted for a in equation (2). The average liver lecithin specific activity is calculated by dividing the area under the liver lecithin specific activity curve by the duration of the experiment. The equation now becomes

$$\frac{\text{Plasma lecithin specific activity at end of experiment}}{\text{Average specific activity of liver lecithin}} = 1 - e^{-t/t_t}$$

The turnover times obtained in this way are presented in Table IV, the results of which clearly show that *choline does not significantly change the turnover rate of either plasma lecithin or sphingomyelin.*

Even though the values for t_t of these two phospholipides were quite similar, their turnover rates do, however, differ because the concentration of lecithin phosphorus in plasma is approximately 4 times as high as that of sphingomyelin phosphorus.

DISCUSSION

In confirmation of earlier observations of Entenman *et al.* (3), it is shown here that the specific activities of choline-containing phospholipides of liver and plasma are markedly elevated shortly after a single feeding of choline. The present investigation brings to light, however, that nearly all of the rise is caused by lecithins. Increases were also observed in liver sphingomyelin, but, since this lipide constituent constitutes only about 10 per cent of the liver's phospholipides, it contributes little to the rise in the specific activities of the choline-containing phospholipides of the liver.

In those animals that responded to choline, the latter produced an increase in the calculated turnover of *liver* lecithins. But in neither *plasma* lecithin nor *plasma* sphingomyelin was an increase in the calculated turnover rates observed after the administration of choline. The increases in the specific activities of plasma phospholipide previously reported (18) must now be recognized as a reflection of similar increases of their precursors in the liver.

The fact that choline stimulates lecithin turnover in the liver but not in plasma may have an important bearing on the mechanism of action of choline as a lipotropic agent. *If the lipotropic action of choline operates through phospholipide metabolism, then the removal of liver fat under the influence of choline does not involve an increased transport of fat from liver to peripheral tissues via plasma phospholipides. The evidence suggests rather that choline acts on the utilization of fat within the liver itself.*[4]

Although the main purpose of this paper was to obtain a liver phosphorus fraction that would enable us to calculate phospholipide turnover in this tissue, it is nevertheless of interest to consider the composition of the alkali-stable fraction. An analysis of this fraction for its glycerophosphate content was made by Burmaster's method (17).[5] In the three normal dogs,

---

[4] Platt and Porter (19) have reported that ethanolamine stimulates cephalin turnover to the same extent as choline stimulates lecithin turnover. Since ethanolamine is not lipotropically active, these authors suggest that the stimulation caused by choline is due to a mass action effect and not to a lipotropic mechanism.

[5] Micromethods for the accurate determination of glycerophosphate in the presence of other organic phosphates have not been developed. The oxidation of glycerophosphate with periodate and the subsequent hydrolysis of the glycolaldehyde phosphate have been used extensively. This method has been criticized, both for its unspecificity (LePage (20)) and its low recovery (86.5 per cent according to Leva and Rapoport (21)). For our purpose, however, the periodate method seemed suitable, since we were mainly interested in learning whether the greater portion of the alkali-stable fraction (Diagram 2) was made up out of glycerophosphate.

66 to 75 per cent of the total P in the alkali-stable fraction was accounted for by glycerophosphate P, whereas 6 to 9 per cent consisted of inorganic phosphate. In the choline-treated animals the percentages for glycerophosphate were 54 to 61, whereas the values for inorganic phosphate were 2, 3, and 11 per cent. These results leave no doubt that the alkali-stable fraction of liver as determined here is rich in glycerophosphate. Since glycerophosphate is structurally an important moiety of the lecithin molecule, and moreover since the specific activity of the alkali-stable fraction satisfied the criteria for a precursor of lecithin, it is likely that glycerophosphate[6] is actually a precursor of liver lecithin.

In an attempt to obtain more direct evidence on the nature of the precursor of liver lecithin, a sample of radioactive glycerophosphate was prepared and injected into the portal vein of a dog at a constant rate during a period of 2.5 hours. At the end of this interval a liver sample was taken. The analyses showed that the specific activity of the inorganic phosphate in the liver was just as high as that of the alkali-stable fraction. It was therefore not possible, from this experiment, to draw more definite conclusions as to whether glycerophosphate is a precursor of liver lecithin.

Since Stetten (22) found that ethanolamine can be considered a precursor of choline, it may be noted that in the present experiments the specific activities of lecithin P in the liver were higher than those of cephalin P. This means that liver cephalin is not on the main pathway of lecithin synthesis and suggests that bound ethanolamine, in contrast to its free form, is not methylated to an appreciable extent.

SUMMARY

1. The specific activity-time relations of liver lecithin, liver cephalin, and liver sphingomyelin were compared with those of two acid-soluble fractions, namely alkali-stable and alkali-hydrolyzable phosphorus.

2. The specific activity-time relations of a liver fraction rich in glycerophosphate met the requirements for a lecithin precursor.

3. Choline increased the turnover of liver lecithin but not of plasma lecithin or of plasma sphingomyelin.

4. In view of these findings it is proposed that choline does not act by increasing fat transport via plasma phospholipides but rather by stimulating the utilization of fats within the liver itself.

BIBLIOGRAPHY

1. Entenman, C., Chaikoff, I. L., and Zilversmit, D. B., *J. Biol. Chem.*, **166**, 15 (1946).

---

[6] The glycerophosphate in the alkali-stable P fraction of the liver could have been derived, in part at least, from glycerophosphorocholine. In the presence of strong alkali the choline radical is readily split off from glycerophosphorocholine.

2. Welch, A. D., and Landau, R. L., *J. Biol. Chem.*, **144**, 581 (1942).
3. Entenman, C., Chaikoff, I. L., and Friedlander, H. D., *J. Biol. Chem.*, **162**, 111 (1946).
4. Zilversmit, D. B., Entenman, C., and Fishler, M. C., *J. Gen. Physiol.*, **26**, 325 (1943).
5. Fishler, M. C., Entenman, C., Montgomery, M. L., and Chaikoff, I. L., *J. Biol. Chem.*, **150**, 47 (1943).
6. Chargaff, E., *J. Biol. Chem.*, **142**, 505 (1942).
7. Flock, E. V., and Bollman, J. L., *J. Biol. Chem.*, **156**, 151 (1944).
8. Riley, R. F., *J. Biol. Chem.*, **153**, 535 (1944).
9. Kurssanov, A., *Biokhimiya*, **3**, 467 (1938).
10. Zilversmit, D. B., Entenman, C., Fishler, M. C., and Chaikoff, I. L., *J. Gen. Physiol.*, **26**, 333 (1943).
11. Taurog, A., Entenman, C., Fries, B. A., and Chaikoff, I. L., *J. Biol. Chem.*, **155**, 19 (1944).
12. Taurog, A., Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, **156**, 385 (1944).
13. Schmidt, G., Benotti, J., Hershman, B., and Thannhauser, S. J., *J. Biol. Chem.*, **166**, 505 (1946).
14. King, E. J., *Biochem. J.*, **26**, 292 (1932).
15. Folch, J., and Van Slyke, D. D., *Proc. Soc. Exp. Biol. and Med.*, **41**, 514 (1939).
16. Chargaff, E., *J. Biol. Chem.*, **144**, 455 (1942).
17. Burmaster, C. F., *J. Biol. Chem.*, **164**, 233 (1946).
18. Friedlander, H. D., Chaikoff, I. L., and Entenman, C., *J. Biol. Chem.*, **158**, 231 (1945).
19. Platt, A. P., and Porter, R. R., *Nature*, **160**, 905 (1947).
20. LePage, G. A., *J. Biol. Chem.*, **152**, 593 (1944).
21. Leva, E., and Rapoport, S., *J. Biol. Chem.*, **149**, 47 (1943).
22. Stetten, D., Jr., *J. Biol. Chem.*, **140**, 143 (1941).

THE TURNOVER RATES OF PLASMA LECITHIN AND PLASMA SPHINGOMYELIN AS MEASURED BY THE DISAPPEARANCE OF THEIR RADIOACTIVE PHOSPHORUS FROM THE CIRCULATION

By D. B. ZILVERSMIT,* C. ENTENMAN, and I. L. CHAIKOFF

(From the Division of Physiology, University of California Medical School, Berkeley)

(Received for publication, May 3, 1948)

Dog plasma contains only two types of phospholipides, namely lecithins and sphingomyelins (1). This is worthy of note, since plasma phospholipides originate in the liver (2), a tissue that synthesizes three types of phospholipides, cephalins as well as those containing choline. The data obtained in the preceding paper (3) showed that lecithin is delivered to plasma at a much more rapid rate than sphingomyelin. Since this measurement was based on a procedure that involved considerable surgical trauma of the liver, the values obtained could not be considered entirely accurate without further proof. A measure of their turnover in plasma was therefore sought by a different method; *i.e.*, one that would cause no physiological disturbance. This was accomplished by introducing labeled plasma phospholipides into the circulation of dogs and observing the rates of their disappearance from the blood stream (4). In contrast to this method, which measures the rate of disappearance of plasma phospholipides, the procedure used in the preceding report was based on the rate at which phospholipides are delivered by the liver to plasma. Since in the steady state the amount of each phospholipide that enters the plasma must equal that which leaves, the two measurements should yield identical results. The close agreement shown here for rates of turnover of each plasma phospholipide as measured by two widely differing methods lends support to the reliability of the data presented.

EXPERIMENTAL

The methods used for the determination of lecithin and sphingomyelin P^{31} and P^{32} are described in the previous communication (3).

In Experiment 1 radioactive phospholipides were obtained from a donor dog that had received orally 4 millicuries of P^{32} *24 hours* before exsanguination. Enough blood was obtained from this donor dog to permit an injection of 100 cc. of plasma into each of three recipient dogs designated here as Dogs A, B, and C. The data for the specific activities of lecithin and sphingomyelin phosphorus of plasma found at various intervals after the injection of radioactive phospholipides are presented in Figs. 1 and 2.

---

* Fellow of the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

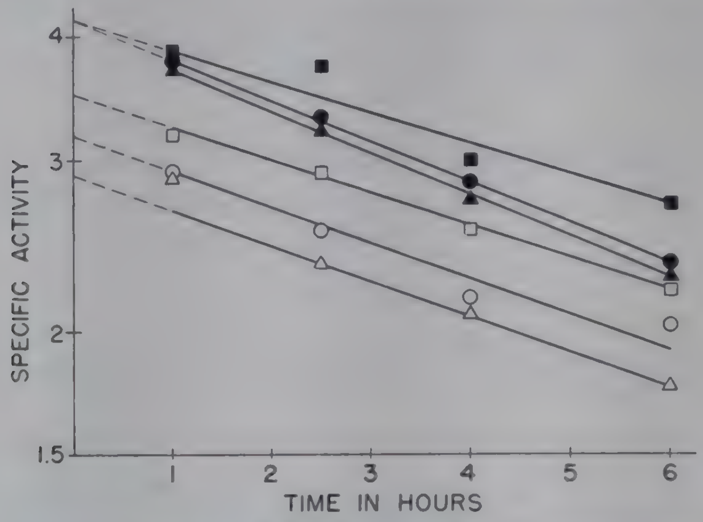


Fig. 1. The disappearance of labeled lecithin from the circulation. Dog A, Experiment 1 (○), Experiment 2 (●); Dog B, Experiment 1 (□), Experiment 2 (■); Dog C, Experiment 1 (△), Experiment 2 (▲).

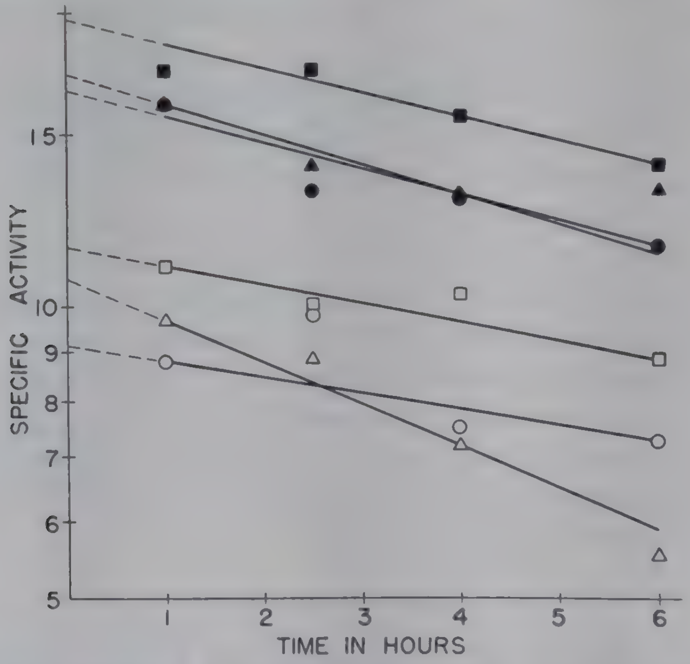


Fig. 2. The disappearance of labeled sphingomyelin from the circulation. Dog A, Experiment 1 (○), Experiment 2 (●); Dog B, Experiment 1 (□), Experiment 2 (■); Dog C, Experiment 1 (△), Experiment 2 (▲).

Calculation of Turnover Time and Turnover Rate

It has been shown in a previous communication (4) that

$$\frac{x}{r} = \frac{x_0}{r} e^{-\frac{p}{r}t} \tag{A}$$

where x is the amount of phospholipide P^{32} present in the entire circulating fluid at time t, x_0 is the amount of phospholipide P^{32} present in the entire circulating fluid at zero time, r is the total amount of phospholipide P present in the entire circulating fluid, p is the rate of phospholipide turnover, and t is the time after injection of the labeled phospholipide.

Thus x/r represents the specific activity of phospholipide P at time t. Since r/p is equal to the time required for the turnover of an amount of phospholipide P equal to that present in the circulating fluid,

$$\frac{x}{r} = \frac{x_0}{r} e^{-t/t_t} \tag{B}$$

where t_t is the phospholipide turnover time.

TABLE I

Turnover Times and Turnover Rates of Plasma Lecithin and Sphingomyelin

Dog	Experiment No.	Weight	Lecithin P	Sphingomyelin P	Turnover time		Turnover rate	
					Lecithin	Sphingomyelin	Lecithin	Sphingomyelin*
		kg.	*mg. per cent*	*mg. per cent*	*hrs.*	*hrs.*	*mg. P per hr.*	*mg. P per hr.*
A	1	7.9	10.6	2.7	12	27	4.3	0.5
	2	8.0	8.7	1.9	12	17	5.6	0.7
B	1	8.8	10.3	2.1	12	23	4.0	0.4
	2	8.9	11.3	2.7	10	17	4.4	0.8
C	1	7.9	10.1	2.0	13	10	4.3	1.0
	2	8.5	9.9	1.7	10	14	5.3	0.7

* The estimation of the total amount of sphingomyelin present in the entire circulating fluid has been made on the basis of the lecithin specific activity data. Thus it is assumed that the circulating volumes for lecithin and sphingomyelin are identical.

Thus,

$$\ln \frac{x}{r} = \ln \frac{x_0}{r} - \frac{t}{t_t} \tag{C}$$

In Figs. 1 and 2 the specific activity of phospholipide P at zero time, *i.e.* x_0/r, can be read from the intersection of the curve with the y axis. If we define the half time ($t_{\frac{1}{2}}$) as the time at which the phospholipide specific activity is exactly half the specific activity at zero time, then we can derive from equation (C) the relation

$$t_t = \frac{t_{\frac{1}{2}}}{\ln \frac{x_0}{x_{\frac{1}{2}}}} = \frac{t_{\frac{1}{2}}}{\ln 2} = 1.44\, t_{\frac{1}{2}}$$

The turnover time is thus easily determined from the half time of any one of the curves in Figs. 1 or 2. The turnover rate can be calculated if the total amount of phospholipide P in the circulation (r) is known.

x_0 is the total amount of phospholipide P^{32} present in the circulation at a time when none of it has yet been utilized and is therefore equal to the total amount of phospholipide P^{32} injected. Dividing the amount of injected phospholipide P^{32} by the specific activity of the phospholipide P at zero time thus gives the total amount of phospholipide P present in the circulation (r).

The turnover rate, p, is then found from

$$p = \frac{r}{t_t}$$

The values for turnover times and turnover rates are recorded in Table I.

The same dogs were used in Experiment 2 carried out 3 weeks later. A new donor dog was used, however; it received orally 4 millicuries of P^{32} *48 hours* before it was bled. The good agreement between the results of both experiments (Table I) shows that the turnover times are not influenced by variables in donor dogs and the time allowed for the syntheses of labeled plasma phospholipides.

It is shown here that the turnover times for plasma lecithin are a little shorter than those for plasma sphingomyelin. Since, however, the plasma of these dogs contained about 4 to 5 times as much lecithin as sphingomyelin, the absolute amounts of these plasma phospholipides turned over (*i.e.*, their turnover rates) differ considerably. Thus, plasma lecithin is turned over at a rate which is more than 5 times as great as that of plasma sphingomyelin.

SUMMARY

The rates of turnover of plasma lecithin and of plasma sphingomyelin have been measured in the dog. The rate for lecithin is more than 5 times as great as that for sphingomyelin.

BIBLIOGRAPHY

1. Taurog, A., Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, **156**, 385 (1944).
2. Fishler, M. C., Entenman, C., Montgomery, M. L., and Chaikoff, I. L., *J. Biol. Chem.*, **150**, 47 (1943).
3. Zilversmit, D. B., Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, **176**, 193 (1948).
4. Zilversmit, D. B., Entenman, C., Fishler, M. C., and Chaikoff, I. L., *J. Gen. Physiol.*, **26**, 333 (1943).

ESTIMATION OF ARGINASE ACTIVITY IN HOMOGENATES*

By EUGENE ROBERTS†

(*From the Barnard Free Skin and Cancer Hospital, St. Louis*)

(Received for publication, June 11, 1948)

The present procedures were developed prior to a study of the changes in arginase activity in epidermal carcinogenesis because none of the existing methods was found to meet the minimal requirements for a good assay (1). Recent advances in the determination of arginase activity of purified liver extracts (2) and in the knowledge of metallic activation of arginase (3) have served as the basis for the modifications described herein.

EXPERIMENTAL

Basic Procedure for Determination of Arginase Activity—A 2 per cent homogenate of the tissue to be studied was prepared in ice-cold redistilled water in a ground glass homogenizer. One aliquot of the above was mixed with an equal volume of a 0.1 M solution of $MnCl_2 \cdot 4H_2O$ and placed in a water bath at 50° in tubes which were tightly stoppered after temperature equilibrium had been established. Samples were removed at the desired intervals for measurement of activity. The tubes were shaken vigorously every 30 minutes. The method for the measurement of arginase activity was essentially that described under "Photometric method" by Van Slyke and Archibald (2). The activity of the original homogenate was measured by adding 0.1, 0.2, or 0.3 ml. of the homogenate or a suitable dilution thereof to 5 ml. test-tubes containing 0.5 ml. of arginine substrate to which 0.9, 0.8, or 0.7 ml. of redistilled water or of 0.05 M $MnCl_2$ had previously been added. The activities of the samples incubated with $MnCl_2$ at 50° were measured only in tubes containing the Mn^{++} ion. The tissue samples were added after the substrate solutions had attained the temperature of the bath (38°). The pH of the mixture containing distilled water as the diluent was approximately 9.5, while that of the mixture containing $MnCl_2$ was approximately 9.2. No differences in activity were observed when samples of the original homogenates were added directly to the Mn-containing incubation mixture or when the homogenates were made 0.05 M with respect to $MnCl_2$ prior to the addition. This is because the Mn^{++} was present in considerable excess. The reaction was allowed to proceed for exactly 10 minutes and

---

* Aided by grants from the United States Public Health Service and the Charles F. Kettering Foundation.

† Present address, Division of Cancer Research, Department of Anatomy, Washington University, St. Louis 10, Missouri.

then stopped by the addition of 1 ml. of 15 per cent metaphosphoric acid. The urea content was measured in the manner previously described (2). A reagent blank was prepared by adding 1 ml. of the acid to the substrate solution to which 1 ml. of water or 1 ml. of 0.05 M $MnCl_2$ had been added. The Mn^{++} ion did not affect the blank value. A tissue blank was also prepared by adding the metaphosphoric acid to the substrate solution prior to the introduction of 0.1 to 0.3 ml. of the homogenate. Liver homogenates were always diluted to 1:10 or 1:20 with redistilled water or with 0.05 M $MnCl_2$ just prior to the determination of activity.

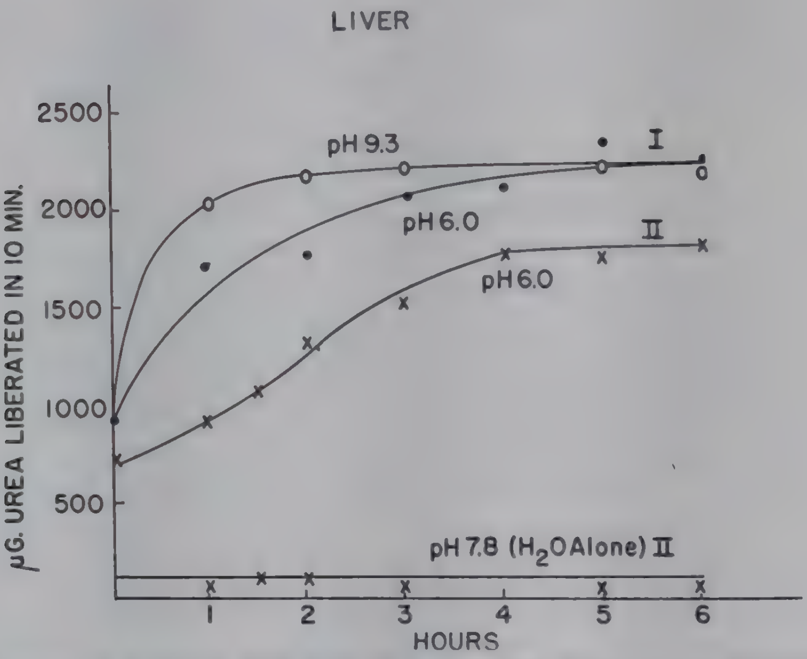


FIG. 1. Change of activity with time of mouse liver homogenate incubated at pH 6.0 or 9.3 in 0.05 M $MnCl_2$ or in water alone at pH 7.8.

Tissues Studied—Special attention was given to the applicability of the method to the determination of the arginase activity of the liver, kidney, and epidermis of the Swiss mouse and of a transplantable squamous cell carcinoma originally derived from the epidermis of a mouse by the application of methylcholanthrene (4). A survey was made of some of the other tissues. The influence of the daily injection of 75 mg. of arginine on the arginase activity of the liver, kidney, and epidermis of adult mice was also investigated.

Results

Activation by Manganese—The results of the study of the time course of manganese activation at 50° are summarized in Figs. 1 and 2 and Table I.

Liver (*Fig. 1*)—From the results of Experiment I it is seen that the maximal activity attained was the same whether the activation took place in an unbuffered solution at pH 6.0 or in 0.25 M glycine buffer at pH 9.3. The rate of activation was somewhat greater at the higher pH. These findings are similar to those previously reported for purified beef liver arginase (3). The incubation in the unbuffered solution was employed routinely because of greater convenience and for the sake of maintaining uniformity with the procedure employed for the other tissues.

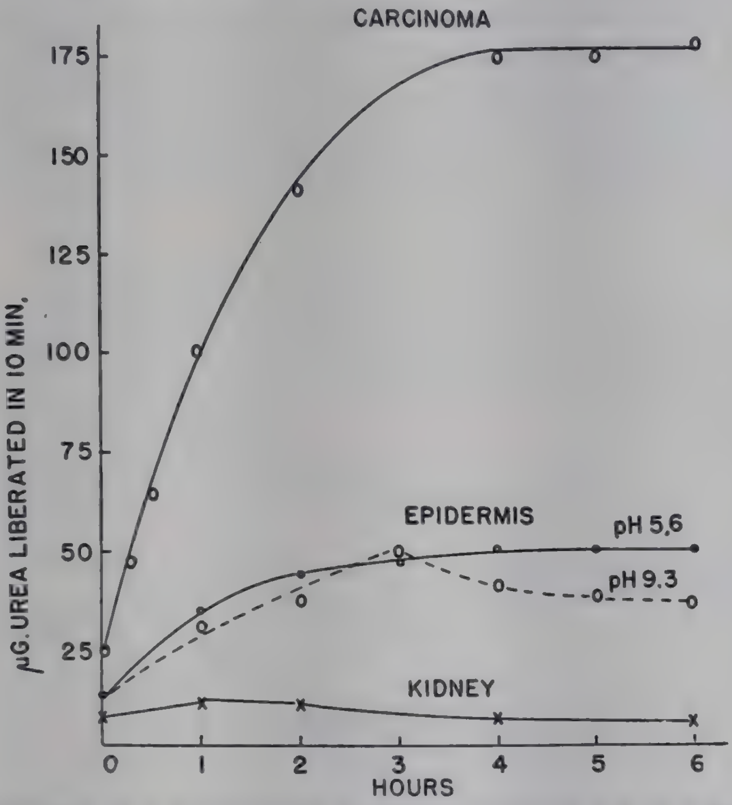


FIG. 2. Activation of a homogenate of mouse epidermis in 0.05 M $MnCl_2$ at pH 5.6 and 9.3 and the activation of homogenates of squamous cell carcinoma and kidney in 0.05 M $MnCl_2$.

Experiment II was performed on another liver sample. The activity attained a maximal level in 4 hours and remained constant through 6 hours as in the comparable sample in Experiment I. In both cases the maximal activity was approximately 2.5 times the activity of the freshly prepared homogenate when estimated in the presence of Mn^{++} ions. Incubation with water alone at 50° produced no activation. The unactivated homogenate had a 5-fold greater activity when the measurements were carried out in the presence of $MnCl_2$ than when no Mn^{++} ions were added.

A 5 hour incubation period was employed for all routine assays. In several experiments it was found that maximal activation was not achieved by heating to 58° for 20 minutes with $MnCl_2$, a procedure found suitable for purified arginase preparations (2).

Epidermis (*Fig. 2*)—Incubation either at pH 5.6 or at pH 9.3 resulted in the same value for maximal activity. This value was attained at the lower pH in 4 hours and remained virtually constant for 6 hours. However, in the alkaline glycine buffer the activity declined after reaching the maximal level at 3 hours. The activation of this tissue was therefore performed routinely in the unbuffered solution for 5 hours at 50°. The maximal activity was approximately 5 times greater than that in the fresh homogenate when the measurements were performed in the presence of Mn^{++} ions.

Carcinoma (*Fig. 2*)—The activity for carcinoma reached a maximum 4 hours after the start of incubation in an unbuffered mixture and remained constant through 6 hours. These findings are the same as for the two previously discussed tissues. The extent of activation achieved was greater in this tissue than in liver and epidermis, the ratio of final to initial activities in the presence of Mn^{++} ions being approximately 7:1. The 5 hour incubation was employed for all assays of carcinoma samples.

TABLE I

Arginase Activity of Various Tissues of Mouse

The results are expressed in micrograms of urea liberated per mg. of fresh weight under the standard assay conditions.

Time of incubation	Tissue			
	Intestine	Lymph nodes	Leg muscle	Spleen
hrs.				
0. No $MnCl_2$	130	1	1	1
0. With $MnCl_2$	111	1	1	1
1	6	1	1	1
2	2		1	1
3	4	3	1	1
4	2	3	1	1
6	14	4	2	1

Kidney (*Fig. 2*)—The results of heating unbuffered homogenate of kidney in 0.05 M $MnCl_2$ at 50° are typical of those obtained in a number of similar experiments. There was a small increase in activity after 1 hour and a slow rate of decrease thereafter. The activity of kidney homogenates was therefore usually measured after 1 hour of incubation. In a few instances no activation was observed.

Other Tissues (*Table I*)—The addition of $MnCl_2$ to the substrate decreased the high activity of the homogenate of washed small intestine to 85 per cent of that found when no activator was added. Most of the remaining activity was lost after incubation for 1 hour at 50°. These results differ from those found for the other tissues studied and merit further investigation. Only slight activity was observed in lymph nodes, leg muscle, and spleen. No activity was detected in heart, pancreas, stomach, brain, testes, whole blood, or thrice-washed erythrocytes in the presence or absence of added $MnCl_2$ or after various intervals of incubation with Mn^{++}. It should be noted that the failure to find arginase activity in these latter tissues does not necessarily mean that they do not possess some arginase activity. Different treatments and the utilization of higher tissue concentrations may be required for the demonstration.

Proportionality of Arginase Activity to Quantity of Tissue—In Fig. 3 are shown the results of experiments in which different quantities of maximally activated preparations of carcinoma, epidermis, and liver were tested under the standard assay conditions. In each case the activity was proportional to the tissue concentration when the quantities of tissue were suitably chosen. Measurements of the activity of an unactivated homogenate of kidney made in the presence of Mn^{++}, Fig. 4, show that also for this tissue the linearity holds true.

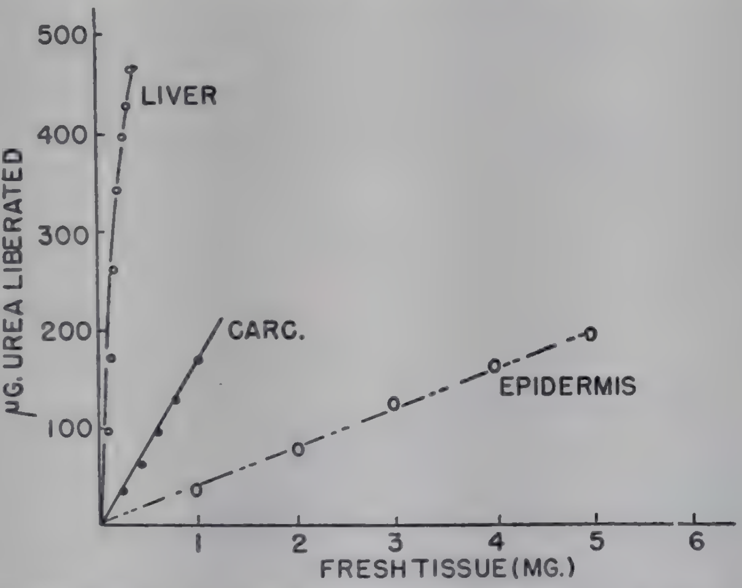


FIG. 3. Proportionality of activity to quantity of tissue in liver, carcinoma, and epidermis.

Time Course of Liberation of Urea—The results of a kinetic study of the liberation of urea from arginine by maximally activated homogenates of epidermis, liver, and carcinoma are given in Fig. 5. In each case 2 ml. aliquots of a suitable dilution of the homogenate in 0.05 M $MnCl_2$ were added to a solution containing 8 ml. of 0.05 M $MnCl_2$ and 5 ml. of the arginine solution to give the final tissue concentrations indicated on the curves. The reactions were carried out at 38°. At various time intervals 1 ml. samples were withdrawn and pipetted into tubes containing 0.67 ml. of 15

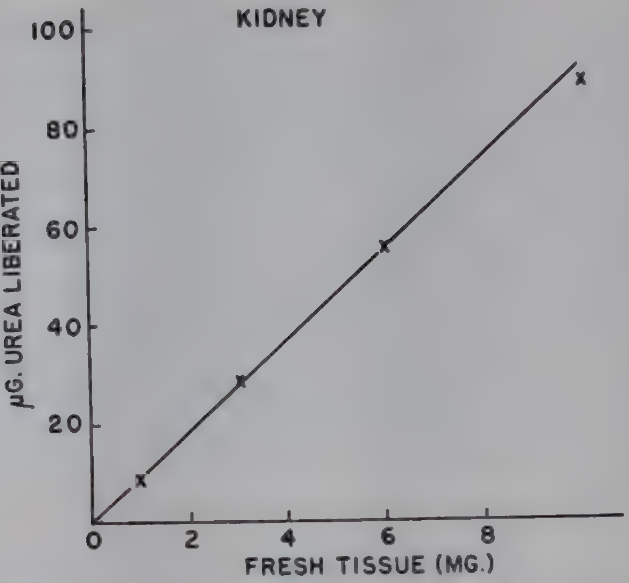


Fig. 4. Proportionality of activity to quantity of tissue in kidney

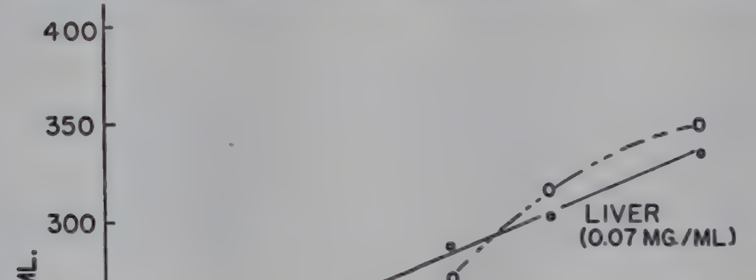


Fig. 5. Time course of liberation of urea by maximally activated homogenates of liver, carcinoma, and epidermis.

per cent metaphosphoric acid. After centrifugation 1 ml. samples of the supernatant fluid were employed for urea determination.

For each tissue the reaction rate was constant over considerably longer periods than the 10 minutes employed in the standard assay procedure.

Typical Assay Results (*Table II*)—An increase in the arginase activity of homogenates of all four tissues was produced by the addition of Mn^{++} ions. A further increase occurred when the tissues were activated at 50°. The activity was proportional to the quantity of tissue employed in all instances except in the case of the unactivated liver homogenate, the activity of which was measured in the absence of added activator. Little importance is attached to the determination of the activity of the homogenate in the absence of added $MnCl_2$ because of the different dilutions of the tissues employed and because of possible contamination with activating ions, especially in the case of the epidermis.

TABLE II

Typical Results for Mouse Liver, Kidney, Epidermis, and Squamous Cell Carcinoma

The results are expressed in micrograms of urea liberated per mg. of fresh weight of tissue under the standard assay conditions.

Treatment	Quantity of homogenate*	Tissue			
		Liver	Carcinoma	Epidermis	Kidney
	ml.				
No $MnCl_2$, no incubation	0.1	280	3	2	6
	0.3	333	3	2	6
With $MnCl_2$, no incubation	0.1	1088	21	7	9
	0.3	1093	18	7	9
Incubated in $MnCl_2$	0.1	2190†	158†	43†	13‡
	0.3	2094	159	46	12

* 0.1 ml. of liver homogenate contained 0.05 mg. of fresh tissue; 0.1 ml. of the homogenates of the other tissues contained 1 mg. of fresh tissue.

† Incubated for 5 hrs. at 50°.

‡ Incubated for 1 hr. at 50°.

Reproducibility of Results—A 2 per cent homogenate was made from epidermis of normal mice and was divided into four aliquots. Each aliquot was studied in the manner previously described. At each level of activation the measurements on the separate aliquots were in excellent agreement with each other.

Summary of Assay Results for Liver, Kidney, Epidermis, and Carcinoma (*Table III*)—The liver has the greatest activity of the tissues studied with the carcinoma, epidermis, and kidney following in that order. The smallest degree of activation by the procedures employed was found in the kidney; the greatest activation was observed in the carcinoma.

Influence of Injection of Arginine on Arginase Activity of the Liver, Kidney, *and Epidermis* (*Table IV*)—An experiment was performed in which 75 mg. of arginine (pH 7.0) contained in 0.5 ml. were injected daily into adult mice, while control mice were given the same quantity of physiological saline. The mice were killed at the times indicated. Only the values for the maximally activated homogenates are reported. It is apparent that the arginine injections produced no significant effect on the arginase activity of the tissues studied. It has been reported that the injection of arginine markedly increased the arginase content of the muscles and kidneys of normal guinea pigs (5). The differing results for the kidneys may possibly be

TABLE III

Arginase Activity of Mouse Liver, Kidney, Epidermis, and Squamous Cell Carcinoma

The results are expressed in micrograms of urea liberated per mg. of fresh weight of tissue under the standard assay conditions. All activities were measured in the presence of Mn^{++} ions.

Sample No.	Tissue							
	Liver		Carcinoma		Epidermis		Kidney	
	Before incubation	After incubation*	Before incubation	After incubation*	Before incubation	After incubation*	Before incubation	After incubation†
1	1179	2740	19	159	7	45	8	12
2	1090	2142	10	79	4	42	9	10
3	935	2330	29	220	5	31	13	16
4	1033	2490	22	206	5	21	9	9
5	1240	2565	24	191	2	22	3	6
Mean....	1095	2453	21	171	5	32	8	11

* After incubation for 5 hrs. at 50°.

† After incubation for 1 hr. at 50°.

TABLE IV

Influence of Injection of Arginine on Arginase Activity of Liver, Kidney, and Epidermis

The results are expressed in micrograms of urea liberated per mg. of fresh weight of tissue under the standard assay conditions. Liver and epidermal homogenates were incubated with $MnCl_2$ for 5 hrs. at 50°; kidney for 1 hr.

Tissue*	Days after first injection	Control†	Arginine‡
Liver....................	6, 7, 10	2231 (1928–2740)	2013 (1587–2676)
Epidermis.................	6, 7, 8	40 (20–57)	40 (35–53)
Kidneys...................	6, 7, 10	10 (6–13)	12 (9–13)

* Four samples were studied for each tissue.

† Received daily intraperitoneal injections of 0.5 ml. of physiological saline.

‡ Received daily intraperitoneal injections of 75 mg. of arginine contained in 0.5 ml. of a solution adjusted to pH 7.0.

ascribed to the different species employed or to variations in experimental procedures.

DISCUSSION

The type of metallic activation that occurs in the case of arginase has also been described for leucine aminopeptidase (6, 7) and prolidase (8). It has been suggested that the activation of arginase consists in the transformation of a proarginase which contains no Mn into an active arginase containing Mn (3). However, the mechanism of the transformation is still not clear. There is no way of estimating the actual activity of the enzyme in the active tissue prior to homogenization and dilution. Therefore, little can be said regarding the physiological significance of determinations made in homogenates until these measurements are correlated with other variables to which they may be related.

It was found that different degrees of activation could be achieved for the various tissues studied. It is important to measure the maximal activity of which a tissue is capable, since this gives an idea of the potentialities of a tissue with respect to the reaction which the enzyme catalyzes. It will be shown in a subsequent report on changes in arginase activity in epidermal carcinogenesis that the most consistent results and those best correlated with other chemical changes were obtained in the maximally activated tissues.

From the results obtained it is apparent that any procedure employed for the measurement of the arginase activity of a tissue must be standardized for that tissue. It would not even seem advisable to transfer a procedure developed for an organ of one species to the same organ of another species without further testing.

SUMMARY

1. A procedure for the determination of the arginase activity of homogenates of tissue was developed from previously reported knowledge of the behavior of this enzyme and was applied to the estimation of the activity of the liver, kidney, and epidermis of the Swiss mouse and of a transplantable squamous cell carcinoma originally derived from the epidermis by the application of methylcholanthrene.

2. The arginase activity of dilute homogenates of the above tissues was increased by the addition of Mn^{++} and was further increased by heating at 50° in the presence of Mn^{++}.

3. The activity was proportional to the quantity of tissue employed and the rate of the reaction was constant under suitably chosen conditions.

4. The liver had the greatest activity, with the carcinoma, epidermis, and kidney following in that order.

5. The injection of arginine had no significant effect on arginase activity of liver, kidney, or epidermis in the mouse.

BIBLIOGRAPHY

1. Potter, V. R., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **4,** 201 (1944).
2. Van Slyke, D. D., and Archibald, R. M., *J. Biol. Chem.*, **165,** 293 (1946).
3. Mohamed, M. S., and Greenberg, D. M., *Arch. Biochem.*, **8,** 349 (1945).
4. Cooper, Z. K., Firminger, H. I., and Reller, H. C., *Cancer Res.*, **4,** 617 (1944).
5. Edlbacher, S., and Schuler, B., *Z. physiol. Chem.*, **206,** 78 (1932).
6. Smith, E. L., and Bergmann, M., *J. Biol. Chem.*, **138,** 789 (1941).
7. Smith, E. L., and Bergmann, M., *J. Biol. Chem.*, **153,** 627 (1944).
8. Smith, E. L., *J. Biol. Chem.*, **173,** 553 (1948).

THE INACTIVATION OF STREPTOMYCIN BY CYANATE*

By ROBERT J. FITZGERALD

(*From the Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina*)

(Received for publication, June 30, 1948)

The reversal of the bacteriostatic action of streptomycin for certain mycobacteria as well as *Escherichia coli* by urea has been the subject of a preliminary note by Fitzgerald and Bernheim (1). At that time the possibility was considered that urea was being directly assimilated by the organisms. Subsequent work to elucidate the nature of this phenomenon has revealed that the agent which causes the reversal of streptomycin is most probably not urea but some product formed when urea is autoclaved in the medium. The purpose of this paper is to present the experimental evidence for this conclusion as well as information on the possible nature of the active reverser and its mode of action.

Methods

The experiments were done with *Mycobacterium tuberculosis* 607 grown in the Tween medium of Dubos (2). The medium was dispensed in a final volume of 10 cc. in test-tubes 1 inch in diameter and suitable for use in the Evelyn photoelectric colorimeter. The inoculum consisted of 0.5 cc. of a 1:20 dilution of a 48 to 72 hour culture which was adjusted to a standard density prior to dilution. All tests were run at 37°. Ammonia determinations were done by a modified method of Van Slyke and Cullen (3) and urea determinations were done by the method of Ormsby (4). Stock 1 per cent solutions of urea were made in 0.05 M phosphate buffer at pH 7.0 and appropriate dilutions made in the medium prior to sterilization by autoclaving at 122° for 10 minutes. Originally this procedure was followed routinely, since only about 10 to 20 per cent of the urea was decomposed by this treatment.

The streptomycin was added aseptically from suitable dilutions of a stock solution of streptomycin sulfate (Winthrop) in sterile distilled water which contained 100,000 γ of streptomycin per ml. Growth was followed turbidimetrically be means of an Evelyn colorimeter with a No. 660 filter.

EXPERIMENTAL

The effect of various concentrations of urea autoclaved in Dubos medium on the bacteriostatic action of 100 γ per cent of streptomycin is shown in

---

* Aided by a grant from the Duke University Research Council.

Fig. 1. Under the test conditions the growth of *Mycobacterium tuberculosis* 607 is inhibited by 10.0 γ per cent of the drug, while in the presence of 12.0 mg. per cent of urea appreciable growth occurs with 100.0 γ per cent of streptomycin. Subcultures from the urea-streptomycin tubes onto veal infusion-glycerin agar containing various concentrations of streptomycin revealed that the sensitivity of these organisms to the drug was the same as the controls. In other words the organisms growing in the urea-streptomycin tubes had not become streptomycin-fast in spite of the fact that under normal conditions *M. tuberculosis* 607 can rapidly develop resistance to this drug. The urea concentration of the medium decreases with growth of the organisms and, since similar results could be obtained with *Escherichia coli* 6522 which presumably contained no urease, the possibility was considered that urea was being assimilated directly by the organisms. However, it was found that any loss of urea could be accounted for by accumulation of ammonia in the medium. Subsequent studies showed that *M. tuberculosis* 607, *M. tuberculosis* BCG 8240, and the H37RV strain of *M. tuberculosis*, as well as *E. coli* 6522, all possessed definite urease activity.

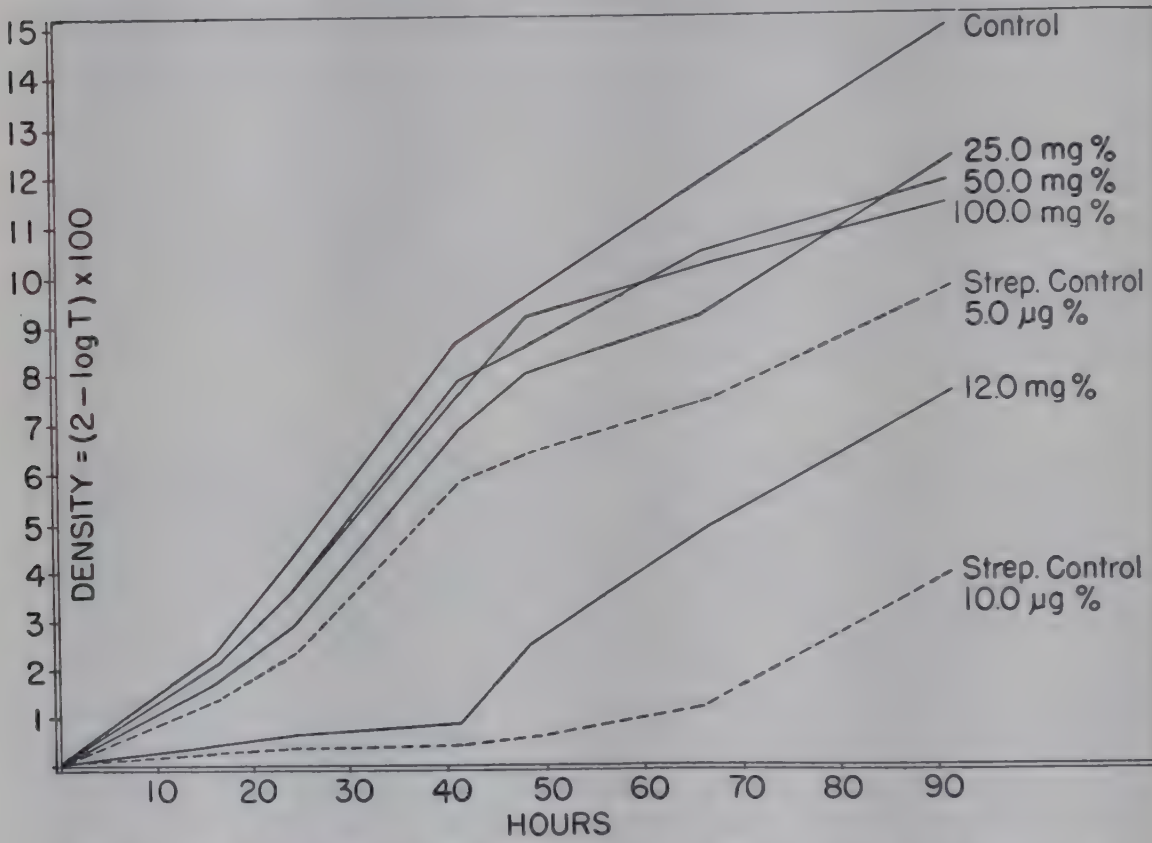


Fig. 1. The effect of urea concentration on the bacteriostasis of *Mycobacterium tuberculosis* 607 by 100.0 γ per cent of streptomycin.

Furthermore it was found that no reversal of streptomycin occurred when the urea was sterilized by filtration through Seitz pads or sintered glass filters and added aseptically to the medium. This would indicate that the active reverser is produced as a result of autoclaving the urea. In order to determine the conditions necessary for the formation of the rereverser, urea was autoclaved separately in distilled water and also together with various components of the medium prior to addition to the test medium. The results of such an experiment are shown in Table I from which it may be seen that the most effective reversal of streptomycin occurs when the urea is autoclaved in the complete medium.

TABLE I

Reversal of Streptomycin by Urea Previously Autoclaved With Different Constituents of Dubos Medium

Supplement*	Streptomycin	Density, $(2 - \log T) \times 100$		
		26 hrs.	42 hrs.	70 hrs.
	γ per cent			
Urea and phosphates	100	0.0	0.0	2.4
	50	0.0	2.4	6.9
“ citrates, and $MgSO_4 \cdot 7H_2O$	100	0.9	1.4	6.7
	50	2.4	6.7	8.3
Urea and complete medium	100	3.1	6.4	8.0
	50	3.4	7.3	8.6
“ “ distilled water	100	0.0	0.0	4.4
	50	0.0	0.0	0.0
Streptomycin control	100	0.0	0.0	0.0
	50	0.0	0.0	0.0
	5	0.0	0.9	7.4
Control	0	2.5	8.8	13.7

* Unless otherwise indicated, each tube contained the equivalent of 100 mg. per cent of urea (before autoclaving). The various supplements as well as the complete medium were autoclaved for 10 minutes at 122°.

To establish somewhat more precisely the conditions for the formation of the reverser samples of the urea-containing medium were boiled for 10 minutes or autoclaved for various periods of time. Neither boiling for 10 minutes nor autoclaving for 1 minute gave rise to any reverser. Best results were obtained when the urea-containing medium was autoclaved for from 5 to 15 minutes. Growth in media with urea autoclaved for longer periods was not optimal, presumably due to the pH changes resulting from accumulation of ammonia.

Removal of the residual urea after it had been autoclaved in the medium was accomplished by addition of purified urease (Squibb). It could be shown after such treatment that the reverser was still present.

A number of compounds that could be considered related to or derived from urea were tested as possible reversers. These included ammonium carbonate, ammonium carbamate, biuret, guanidine hydrochloride, thiourea, sodium cyanide, sodium thiocyanate, and potassium cyanate. All the compounds were made up in 0.05 M phosphate buffer at pH 7.0 and sterilized by Seitz filtration. Additions were made to the test mixtures from appropriate dilutions in Dubos medium. None of these compounds could function as a reverser, with the exception of potassium cyanate.

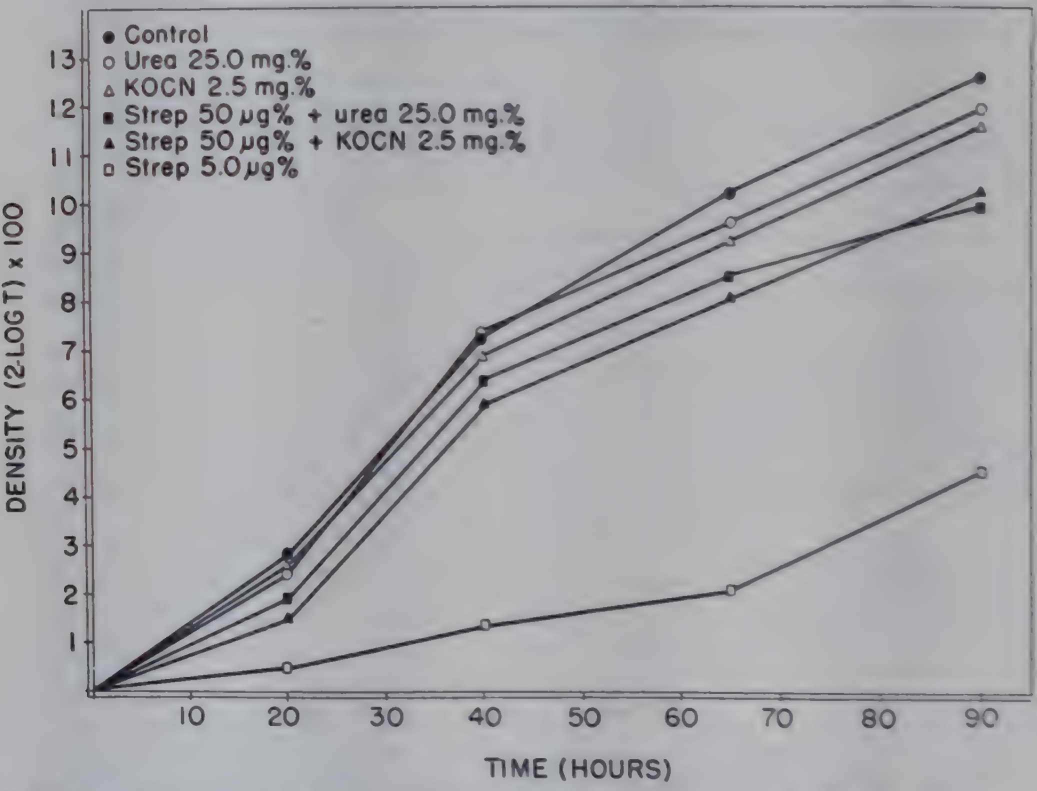


FIG. 2. Comparison of urea autoclaved in the medium and potassium cyanate added aseptically as reversers of streptomycin.

Fig. 2 shows a comparison of potassium cyanate added aseptically and urea autoclaved in the medium as streptomycin reversers. The cyanate has the greater activity, being approximately 10 times as effective as the autoclaved urea in counteracting the bacteriostatic effect of streptomycin on *Mycobacterium tuberculosis* 607. Similar results were obtained with *Escherichia coli* 6522 in the medium of Kohn and Harris (5), and a laboratory strain of *Staphylococcus aureus* in Difco nutrient broth. When the cyanate was autoclaved in the medium most of its ability to reverse streptomycin was lost.

Cyanate is probably not utilized by these organisms because concentrations above 10 mg. per cent inhibit growth. It seemed probable therefore that cyanate was reacting with the streptomycin molecule. If this were so, cyanate added to washed suspensions of mycobacteria should counteract the inhibitory effect of streptomycin on the formation of the adaptive enzyme for benzoic acid (6). Experiments were done with *Mycobacterium tuberculosis* 607 and *Mycobacterium lacticola*. 1.0 mg. of potassium cyanate completely prevented the inhibition of enzyme formation by 0.2 mg. of streptomycin. Since cyanate alone inhibits the oxygen uptake under these conditions it probably is not metabolized. This experiment indicates therefore that cyanate reacts with the streptomycin molecule. Because of the ability of cyanate to react with amino groups, it is probable that it combines with these groups in streptomycin.

DISCUSSION

The inhibition of the bacteriostatic effect of streptomycin by urea autoclaved in Dubos medium is most probably due to formation of some new compound, since urea itself does not possess this property. Salt effects on streptomycin are ruled out because comparable concentrations of ammonium carbonate or ammonium carbamate are inactive. Of the compounds tested potassium cyanate is the only one which meets the requirements of the hypothetical compound in that it reverses streptomycin and small amounts of cyanates may be formed by autoclaving urea solutions.

In view of the fact that cyanate does not seem to be a normal metabolite of *Mycobacterium tuberculosis* 607 and may actually inhibit its growth and metabolic activity it is probable that it reacts with the streptomycin molecule. Although the nature of this reaction has not been elucidated, the reactivity of cyanates with free amino groups and the presence of these groups in the streptamine portion of the molecule suggest that this is the point of attack. Donovick *et al.* (7) have already demonstrated that streptomycin may be inactivated by certain carbonyl reagents such as semicarbazide, thiosemicarbazide, and hydroxylamine which presumably act upon the carbohydrate portion of the molecule. It would appear from the results herein presented that the free amino groups of streptomycin are also essential for antibiotic activity.

SUMMARY

1. When urea is autoclaved in Dubos medium a product is formed which blocks the bacteriostatic action of streptomycin.

2. Of the substances tested cyanate is the only one which satisfies the characteristics of this reverser.

3. Cyanate probably inactivates streptomycin directly, possibly by combining with its free amino groups.

BIBLIOGRAPHY

1. Fitzgerald, R. J., and Bernheim, F., J. Biol. Chem., 172, [illegible] (1948).
2. Dubos, R. J., Davis, B. D., Middlebrook, G., and Pierce, C., Am. Rev. Tuberc., [illegible] (1946).
3. Hawk, P. B., Oser, B. L., and Summerson, W. H., Practical physiological chemistry, Philadelphia, 12th edition (1947).
4. [illegible], J. Biol. Chem., 168, [illegible] (1947).
5. [illegible], and [illegible], J. Pharmacol. and Exp. Therap., [illegible] (1941).
6. Fitzgerald, R. J., and Bernheim, F., J. Bact., 55, [illegible] (1948).
7. [illegible], [illegible], and [illegible], J. Biol. Chem., [illegible] (1948).

E REDUCTION OF COZYMASE BY SODIUM BOROHYDRIDE*

By MARTIN B. MATHEWS

(From the Department of Chemistry, University of Chicago, Chicago)

(Received for publication, June 23, 1948)

ozymase (diphosphopyridine nucleotide, DPN) has been assayed by a ety of biochemical and chemical methods (1). Among the simplest cedures has been the determination of the light absorption of reduced N at 340 mμ after reduction by sodium hydrosulfite. Since special pre- tions are required to remove completely the excess reducing agent which orbs in this region, it appeared advantageous to find another reducing nt which could be employed for the same purpose with greater ease.

study of the action of sodium borohydride ($NaBH_4$) on DPN revealed it filled the necessary requirements. The main advantage of this new gent lies in its absence of light absorption at 340 mμ. Spectrophoto- ric measurement of DPN reduced by $NaBH_4$ may therefore be made out removal of excess reagent. The assay procedure together with e observations on the properties of the reduction product will be ribed in this communication.

EXPERIMENTAL

eagents—Sodium borohydride was obtained as a white powder of roximately 95 per cent purity.[1] Its preparation and some of its proper- have been previously described (2). In water solution, $NaBH_4$ hy- yzes slowly according to the equation $NaBH_4 + 2H_2O \rightarrow Na^+ +$ $^- + 4H_2$.

is reagent was selected from a variety of available metallo hydrides use it shows a moderate stability in water solution. The rate of olysis was determined approximately by titrating $NaBH_4$ with iodine. it was ascertained that a 0.1 per cent solution in distilled water was pletely hydrolyzed in 1 minute at 100°, but that at 4° only about 10 cent of the available reducing ability of a 5 per cent solution was lost day. These properties make it possible to destroy excess reagent letely when this is necessary, and also to prepare the reagent for use olution which may be kept in the cold for several hours.

zymase was purchased from the Schwarz Laboratories. Solid dihy- zymase was prepared according to Ohlmeyer (3). The assay of DPN

---

his investigation was aided by a grant from the Rockefeller Foundation.

[1] indly furnished by Albert Stewart, Chemistry Department, University of go.

with sodium hydrosulfite was carried out according to the procedure used by LePage (4).

All spectrophotometric measurements were made with a Beckman quartz spectrophotometer, model DU.

Assay of DPN with $NaBH_4$—The assay of DPN is accomplished by adding about 2 mg. of $NaBH_4$ (as solid or in solution) to 3.0 cc. of 0.1 M phosphate buffer, pH 7.0, containing about 0.5 mg. of DPN. These conditions are suitable for cells of 1 cm. width commonly used with the Beckman spectrophotometer. About 1 minute is allowed for the reduction. During this time the solution is shaken gently to aid removal of hydrogen formed by hydrolysis of the reagent. The optical density is then measured at once. Interference with the measurement by gas bubbles can readily be avoided by proper tilting of the cuvette immediately before the reading is made. Phosphate buffer may be used as a blank, but corrections must of course be made for any light absorption by impurities in the unreduced DPN preparation.[2]

TABLE I

Assay of Cozymase Preparations

	Per cent purity*	
	Lot 1	Lot 2
By reduction with sodium hydrosulfite (LePage (4))	37.6 ± 0.4	40.5 ± 1.0
" " " " borohydride; spectrophotometric	38.1 ± 0.2	39.9 ± 0.8
By reduction with sodium borohydride; iodine titration	39.1 ± 0.5	41.6 ± 0.5

* Data are given with the average deviation from the mean of two or more determinations.

The validity of the method rests upon the fact that the spectrum of the reduction product is identical with that obtained with sodium hydrosulfite for the region 330 to 400 mμ. The absorption band with a peak at 265 mμ, characteristic also of adenine derivatives present as impurities in the DPN preparations used, was not appreciably changed by $NaBH_4$. Table I shows representative results obtained on assaying two different DPN preparations spectrophotometrically, by means of both the $NaBH_4$ and the hydrosulfite procedures. The percentage purity was calculated from an extinction coefficient of 6.27×10^6 sq. cm. per mole at 340 mμ (3).

---

[2] It is necessary to use a buffer of pH 7.0, since the reduction of DPN by $NaBH_4$ in solutions of pH greater than 7.5 leads to the production of a stable yellow color possessing an absorption peak at 340 mμ and a smaller peak at about 420 mμ. This does not occur at pH 7.0 to 7.1. A stable yellow product has been obtained by Adler and coworkers (5) on reduction of DPN with sodium hydrosulfite in highly alkaline medium. It has commonly been supposed to be a semiquinoid radical (6). The stable yellow substance obtained upon reduction of DPN with $NaBH_4$ in media of pH greater than 7.5 differs considerably from the above product in absorption spectrum and will be reported on more fully in a subsequent communication.

Table I also shows the assay values obtained by iodine titration of the reduced DPN formed by $NaBH_4$. Titration of reduced DPN was carried out with 0.005 N iodine solution and starch as indicator. This method was first suggested by Karrer and Ringier (7) and has more recently been used by Drabkin (8) as a method for assaying solid dihydrocozymase. 1 mole of pure dihydrocozymase is quantitatively oxidized by 2 equivalents of iodine. For the determinations reported in Table I, the reduction was carried out as described for the spectrophotometric assay with a somewhat larger amount of the reactants, and excess $NaBH_4$ was then removed by heating to 100° for 1 minute.

Biological Activity of Product—The enzymatic reoxidation of reduced DPN was carried out with sodium pyruvate and lactic dehydrogenase obtained by dialysis of an extract of a pig heart acetone powder. In no case did enzyme alone or substrate alone produce any reoxidation. Reduced DPN obtained by use of sodium hydrosulfite was found to be 100 per cent reoxidizable by lactic dehydrogenase, as was demonstrated by Green and Dewan (9). Under similar conditions, DPN reduced by $NaBH_4$ was reoxidized only 45 to 51 per cent. Partial enzymatic reoxidation of $NaBH_4$-reduced DPN lowered the absorption band with a peak at 340 mμ in the same proportion at each wave-length over the range of 330 to 400 mμ.

It has been earlier observed that sodium bisulfite and hydrogen cyanide combine with DPN (but not with reduced DPN) to yield addition products absorbing at 340 mμ (10). That there was no interference from comparable addition products in the reoxidation of DPN reduced by $NaBH_4$ follows from the unchanged optical density obtained on the addition of a completely hydrolyzed solution of $NaBH_4$ to DPN.

DISCUSSION

The agreement between assay values appearing in Table I shows that sodium borohydride may be satisfactorily used for the assay of DPN.[3] The products of reduction, however, though closely similar, are not entirely identical to those obtained by the use of sodium hydrosulfite.

The probability that reduction by sodium borohydride produced two isomeric forms of dihydrocozymase should be considered. Since, as pointed out by Karrer and coworkers (11), the two possible isomeric dihydro products would be expected to differ in ultraviolet spectrum, it seems more probable that sodium borohydride acts upon DPN in a more complex manner.

---

[3] In applying $NaBH_4$ to the analysis of triphosphopyridine nucleotide, Mr. Eric Conn of the Department of Biochemistry, University of Chicago, has found that the results are in agreement with those obtained by the reduction of triphosphopyridine nucleotide with glucose-6-phosphate and *Zwischenferment*.

SUMMARY

Sodium borohydride has been applied successfully to the assay of DPN. Since this reagent does not absorb in the near ultraviolet and the excess is readily hydrolyzed to sodium borate, the new assay procedures are more convenient than previous ones with sodium hydrosulfite. However, the reduced products obtained are only partially enzymatically active.

I should like to express my thanks to Professor Birgit Vennesland and to Professor T. R. Hogness for their kind criticism and encouragement during the course of this work.

BIBLIOGRAPHY

1. Schlenk, F., and Schlenk, T., *Arch. Biochem.*, **14**, 131 (1947).
2. Hoekstra, H. R., Dissertation, University of Chicago (1947).
3. Ohlmeyer, P., *Biochem. Z.*, **297**, 66 (1938).
4. LePage, G. A., *J. Biol. Chem.*, **168**, 623 (1947).
5. Adler, E., Hellström, H., and von Euler, H., *Z. physiol. Chem.*, **242**, 225 (1936).
6. Karrer, P., and Benz, F., *Helv. chim. acta*, **19**, 1028 (1936); Hellström, H., *Z. physiol. Chem.*, **246**, 155 (1937).
7. Karrer, P., and Ringier, B. H., *Helv. chim. acta*, **20**, 622 (1937).
8. Drabkin, D. L., *J. Biol. Chem.*, **157**, 563 (1945).
9. Green, D. E., and Dewan, J. G., *Biochem. J.*, **31**, 1069 (1937).
10. Meyerhof, O., Ohlmeyer, P., and Möhle, W., *Biochem. Z.*, **297**, 113 (1938).
11. Karrer, P., Kahnt, F. W., Epstein, R., Jaffé, W., and Ishii, T., *Helv. chim. acta*, **21**, 223 (1938).

CARBOHYDRATE METABOLISM IN HIGHER PLANTS

I. PEA ALDOLASE

By P. K. STUMPF

(From the Division of Plant Nutrition, University of California, Berkeley)

(Received for publication, June 23, 1948)

In recent years muscle aldolase has been studied extensively by several investigators (1–5). The enzyme has been crystallized and its properties and kinetics carefully documented. Little attention, however, has been paid to its counterpart in plant tissue. Although its presence in plants has been indicated by several workers, namely Baba (6), Allen (7), and James *et al.* (8), its properties, kinetics, and biological function have not as yet been analyzed.

The purpose of this communication is to present such data for a plant aldolase isolated from the pea seed. Subsequent communications will deal with the distribution and biological rôle of aldolase in higher plants and other key enzymes involved in carbohydrate metabolism in higher plants.

Preparation and Properties

For the most part pea seeds (Dwarf Telephone) were employed as the source for the enzyme, though squash seeds were also found suitable. The enzyme was readily extracted from pea seeds, which had been soaked for 12 hours in distilled water at 2°, with 0.1 per cent potassium carbonate as the extracting solvent. The extract was then subjected to (1) ammonium sulfate fractionation, (2) isoelectric precipitation with dilute acetic acid at pH 5.5, by which procedure much inert protein was precipitated, while aldolase remained in solution, and finally (3) acetone fractionation followed by dialysis and a final isoelectric precipitation. The details of the purification procedure are summarized in Table I and discussed in the experimental section.

Enzyme activity was measured by the cyanide-fixing method of Herbert *et al.* (1), in which the triose phosphates, formed from the scission of fructose diphosphate, react with cyanide to yield cyanohydrins. Triose phosphate P was then estimated after hydrolysis by exposure to strong alkali for 20 minutes at 20°. The enzyme unit employed in this investigation differed from others, since pea aldolase does not have the high specific activity of its counterpart in animal tissue. Therefore, in establishing a reproducible unit, the amount of alkali-labile P released has been decreased 10-fold, while the time period was increased from 3 to 10 minutes. The unit may then be defined as the amount of enzyme required to liberate

0.1 mg. of inorganic P equivalent to triose phosphate in 10 minutes at 30° in a 0.1 M veronal buffer of pH 8.5 in an excess of fructose diphosphate and cyanide. Other carbonyl fixatives, such as bisulfite, hydrazine, and semicarbazide, in final concentrations equivalent to that of cyanide (0.08 M) can be employed. Hydroxylamine was unsatisfactory because it inhibited aldolase completely in a 0.01 M final concentration.

The final enzyme fraction of highest purity (Fraction Bs) is a water-clear solution with no isomerase activity. Since the initial extract contained 0.06 unit per mg. of protein and the final preparation 5.5 units per mg. of protein, the degree of purification relative to the first extract is 5.5/0.06 or 92-fold. 1 mg. of protein (Fraction Bs) forms 3.3 mg. of triose phosphate P per hour or 18.1 mg. of triose phosphate per hour under standard conditions at 30°.

TABLE I

Purification of Pea Aldolase

Fraction	Purification procedure	Total units	Units per mg. protein
Extract		460	0.06
I	Neutral saturated $(NH_4)_2SO_4$ to 35% saturation	43	0.007
II	" " " " 70% "	341	0.11
	II dialyzed 5 hrs. against distilled water at 50°		
IIa	Neutral saturated $(NH_4)_2SO_4$ to 40% saturation	14	0.03
IIb	" " " " 51% "	98	0.14
IIc	" " " " 61% "	165	0.19
IId	" " " " 70% "	34	0.07
	IIc dialyzed overnight against distilled water		
IIc, residue	Ppt. with 1% acetic acid to pH 5.5	6	0.04
" supernatant (A)		155	0.25
A	Acetone added to 55%	5	0.08
B	" " " 62%	135	0.32
C	" " " 72%	2	
	B dialyzed overnight		
B, residue	Ppt. with 1% acetic acid to pH 5.5	8	0.11
" supernatant (Fraction Bs)		97	5.50

The enzyme is strongly adsorbed on alumina C γ. All attempts to elute with dilute alkaline solutions, including veronal buffers, sodium bicarbonate, and ammoniacal ammonium sulfate, failed. Some attempts were made to induce crystallization by the techniques described recently by Taylor *et al.* (3), but without success.

The enzyme is a rather stable protein. Solutions of the enzyme can be frozen and stored indefinitely without loss of activity. Dialysis in the cold against distilled water resulted in no loss, though dialysis at room temperature did cause appreciable loss. It is unstable below pH 5.5 and above 10, but stable within these ranges. Organic solvents, such as acetone or alcohol, did not alter its stability.

Exposure of a neutral solution of the enzyme for 5 minutes at 45° caused no inactivation. However, at 50° a 4 per cent loss was observed, at 55° a 70 per cent loss, and at 60° complete inactivation occurred.

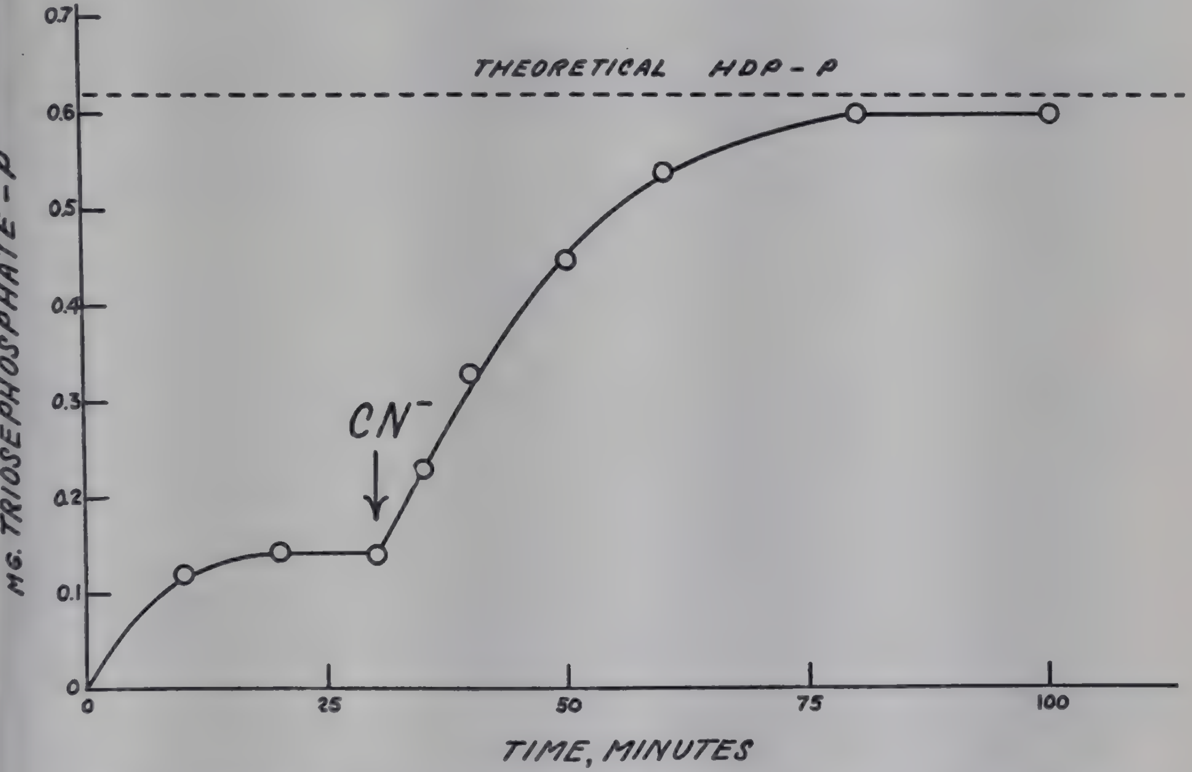


FIG. 1. Effect of time on decomposition of hexose diphosphate. Each tube contained 1.0 cc. of enzyme (Fraction Bs), 0.5 cc. of 0.1 M veronal buffer at pH 8.5, and 0.1 cc. of 0.1 M hexose diphosphate. At a given time 0.1 cc. of 2 M cyanide at pH 8.5 was added. Temperature 31°.

Reaction—Pea aldolase catalyzes the reversible reaction

(1) Fructose diphosphate ⇄ dihydroxyacetone phosphate + D-3-glyceraldehyde phosphate

The reversible nature of this reaction is illustrated in Fig. 1. Equilibrium was attained in approximately 10 minutes. Addition of cyanide to the reaction mixture fixed the triose phosphates and thus the reaction was shifted completely to the right. Within 70 minutes hexose diphosphate was quantitatively converted to an equimolar mixture of dihydroxyacetone phosphate and D-3-glyceraldehyde phosphate.

Iodine oxidation experiments with equilibrium mixtures demonstrated that equimolar mixtures of the triose phosphates were formed. Therefore it may be concluded that isomerase was absent from the enzyme preparations employed in equilibrium studies. Although both triose phosphates are unstable in strong alkali, iodine oxidation converts D-3-glyceraldehyde phosphate, but not dihydroxyacetone phosphate, to the very stable phosphoglyceric acid. Thus, after iodine oxidation of an equilibrium mixture, if no isomerase is present, the alkali-labile phosphate, derived now solely from the degradation of dihydroxyacetone phosphate, should fall to 50 per cent or less of the original alkali-labile phosphate. Experimentally, after iodine oxidation of different equilibrium mixtures, a decrease of from 50 to 70 per cent of the original total alkali-labile phosphate was consistently observed.

The equilibrium constant K was therefore estimated from the equation

$$K = \frac{(\frac{1}{2}\text{ triose phosphate})^2}{(\text{fructose diphosphate})} \tag{2}$$

and was found to be about 1.15 × 10^{-4} mole per liter at 31° (in borate buffer at pH 8.5), which is somewhat higher than that of animal aldolase which is about 0.9 × 10^{-4} mole per liter at 31° (1, 2).[1] As summarized in Table II, the equilibrium is shifted to the right with an increase in temperature and therefore follows the van't Hoff law of reaction isochores.

Pea aldolase like animal aldolase exhibits the same high substrate specificity in that only fructose-1,6-diphosphate is broken down to the triose phosphates. The monophosphates of glucose, glucose-1-phosphate and glucose-6-phosphate, and the monophosphate of fructose, fructose-6-phosphate, are inert in the enzyme system.

The pH optimum, shown in Fig. 2, is approximately 8.5 in 0.1 M veronal buffer. Since the enzyme protein is unstable in the higher as well as in the lower pH ranges (above pH 10 and below pH 5), the fall in activity is due to a destruction of the enzyme protein rather than to a reversible decrease in enzyme activity. In setting up the pH dependence curve, all pH adjustments of reaction mixtures were controlled with the Beckman pH meter.

Figs. 3 and 4 indicate the relation of enzyme activity to enzyme concen-

---

[1] The slight discrepancy between the observed K values for pea aldolase and the values cited for the animal aldolase could not be reconciled despite the following considerations: (*a*) since isomerase is absent, the differences observed could not be caused by K measurements of two reactions, catalyzed by aldolase and isomerase, (*b*) phosphatase is absent since no inorganic P is detected in reaction mixtures, (*c*) though borate buffers at pH 8.5 were employed, buffers of pH 7.3 used by Meyerhof and Herbert gave similar results, (*d*) experiments with muscle aldolase gave K values which checked closely with values in the literature.

TABLE II

Aldolase Equilibrium at Different Temperatures*

Enzyme	Temperature	Time	Hexose diphosphate, final	Triose phosphate formed	*K*	*K* (mean)
cc.	*°C.*	*min.*	$M \times 10^{-3}$	$M \times 10^{-3}$	$M \times 10^{-4}$ *per l.*	$M \times 10^{-4}$ *per l.*
0.5	31	20	3.83	1.40	1.28	
0.5		20	8.03	2.06	1.32	
0.5		40	8.01	2.1	1.37	
0.5		30	6.27	1.42	0.80	
0.5		20	4.07	1.46	1.31	
1.0		20	4.05	1.50	1.39	
0.5		30	3.9	1.2	0.92	1.15
0.5	22	40	3.94	1.18	0.88	
1.0		40	3.92	1.22	0.95	0.91
1.0	40	20	3.23	2.6	5.25	
1.0		40	7.03	4.07	5.87	5.56

* In 0.1 M borate buffer at pH 8.5.

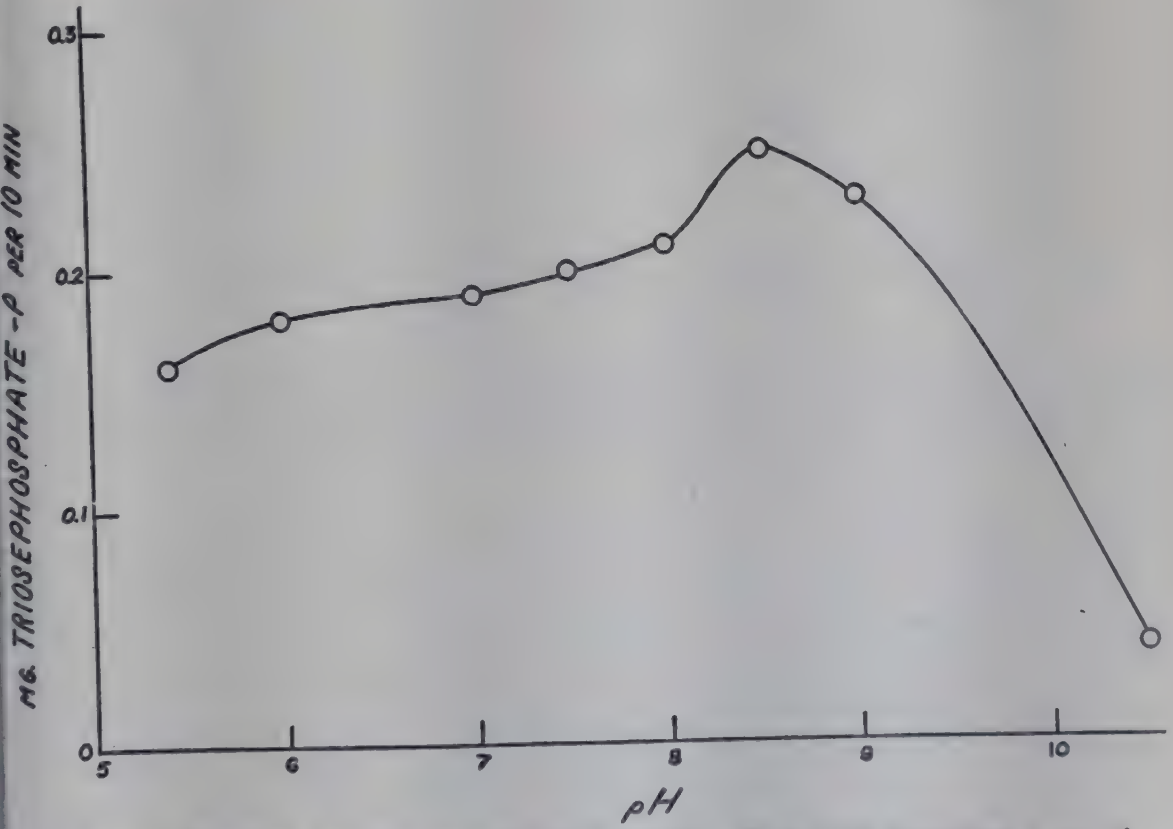


FIG. 2. Reaction velocity as a function of pH. Each tube contained 1 cc. of enzyme, 0.5 cc. of 0.1 M hexose diphosphate, 1 cc. of 0.1 M veronal buffer, and 1 cc. of 0.25 M cyanide, the reaction mixture being adjusted to the desired pH. 0.1 M acetate buffer was employed for the value at pH 5.4. Temperature 31°.

tration and substrate concentration, respectively. From Fig. 4, the K_m is approximately 0.8×10^{-3} mole per liter, which suggests that pea aldolase has an affinity for the substrate some 10-fold greater than that of animal aldolase, which has a K_m of about 9×10^{-3} mole.

In sharp contrast to the animal enzyme (1, 4), pea aldolase is not very sensitive to heavy metal inhibition. Thus in a final concentration of 10^{-4} M, copper sulfate, mercuric acetate, phenylmercuric acetate, and silver nitrate did not inhibit the enzyme. Further, in a final concentration of 10^{-3} M iodoacetamide, fluoride, indoleacetate, and azide as well as a saturated solution of capryl alcohol and 10^{-4} N iodine gave no inhibition. Cysteine, α,α'-dipyridyl, and cyanide, contrary to the observations of Warburg and Christian with yeast aldolase (5), did not inhibit the enzyme.

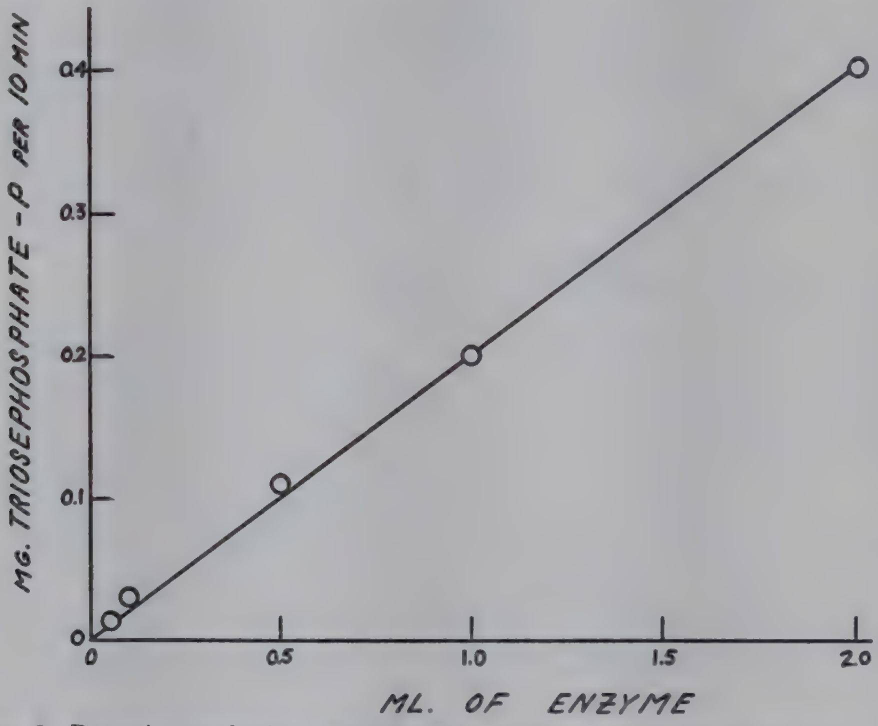


FIG. 3. Reaction velocity as a function of enzyme concentration. Each tube contained 0.5 cc. of 0.1 M hexose diphosphate, 1 cc. of 0.1 M veronal buffer at pH 8.5, 1 cc. of 0.25 M cyanide at pH 8.5, varying concentrations of enzyme, and water to a final volume of 4.5 cc. Temperature 31°.

Several compounds which might be considered as possible competitive inhibitors for the active center of the enzyme were examined. In final concentrations of 10^{-2} M, glucose, fructose, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, β-glycerophosphate, and pyruvic acid did not cause any decrease in the rate of fructose diphosphate decomposition.

Of the several carbonyl reagents tested, cyanide, bisulfite, semicarbazide, hydrazine (adjusted to a final pH of 8.5) in a final concentration of 8×10^{-2}

M were equivalent in their fixative capacity. Hydroxylamine, however, was the only exception, since it acted as an inhibitor of the enzyme. Thus in a final concentration of 10^{-2} M, the reagent inhibited the enzyme 100 per cent; in 10^{-3} M, 55 per cent; and in 10^{-4} M, only 14 per cent. Since divalent cations such as cobalt, zinc, magnesium, and manganese did not reverse the inhibition, the nature of the inhibition was probably not related to the formation of a metal-reagent-enzyme complex.

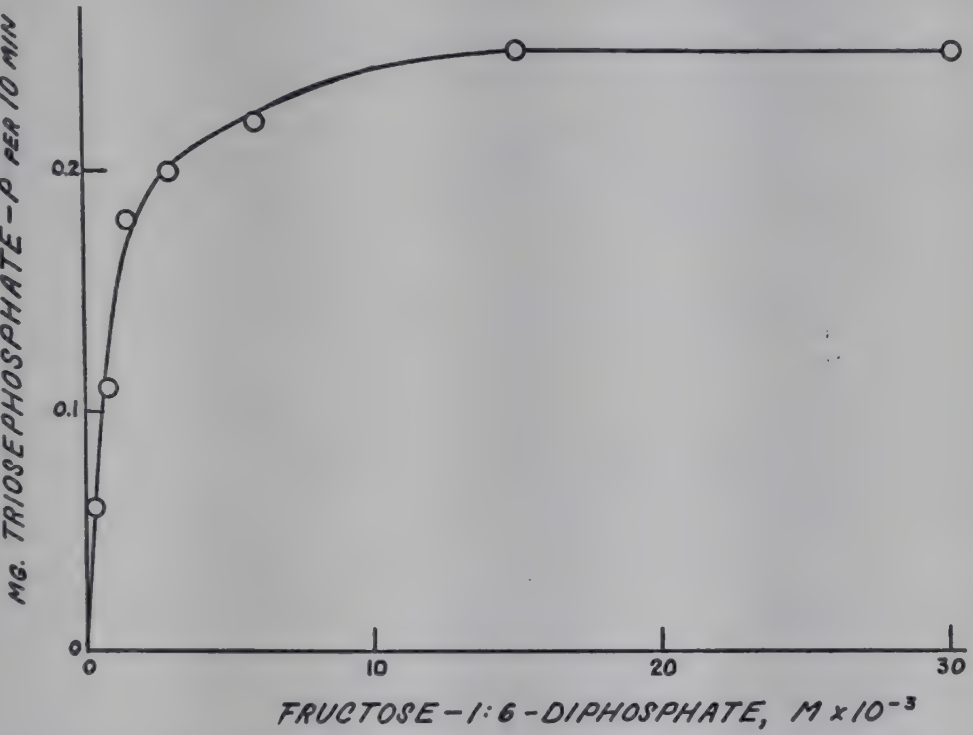


FIG. 4. Reaction velocity as a function of substrate concentration. Each tube contained 0.5 cc. of enzyme, 1 cc. of 0.25 M cyanide, 1 cc. of 0.1 M veronal buffer at pH 8.5, increasing concentrations of 0.1 M hexose diphosphate, and water to a final volume of 3.5 cc. Temperature 31°.

Attempts to resolve the enzyme into a protein moiety and a specific prosthetic group were unsuccessful. After dialysis for 12 hours at 2° against 10^{-2} M acetate buffer at pH 4.5, the enzyme was completely inactive; addition of either zinc, magnesium, manganese, or cobaltous ions to the dialyzed enzyme did not restore activity. After dialysis for the same period of time against 0.05 per cent potassium carbonate, no loss in activity was observed. Prolonged dialysis against distilled water and exposure of the enzyme to saturated ammonium sulfate at pH 4.5 also did not yield successful results.

EXPERIMENTAL

Preparation—In a typical purification procedure, 25 gm. of pea seeds (Dwarf Telephone), after soaking overnight at 2° in an equal volume of distilled water, were suspended in 10 volumes of cold 0.1 per cent potassium carbonate and dispersed in a Waring blendor for 5 minutes. The larger particles were removed by passing the extract through several layers of moist cheese-cloth. Purification procedures were then employed, as outlined in Table I.

In ammonium sulfate fractionation, neutralized saturated ammonium sulfate rather than unneutralized or solid ammonium sulfate was employed and found to give higher yields. Since long periods of gravity filtration of ammonium sulfate precipitation at room temperature resulted in some loss of enzyme, all separations were therefore carried out in the cold by centrifuging in the International refrigerated model No. 1. Rapid isoelectric precipitations with 1 per cent acetic acid at 0° to pH 5.5 were essential, since even short exposure to pH lower than 5.5 led to irreversible inactivation.

All attempts to carry purification beyond the stage of 5.5 units per mg. of protein were discontinued, since it was consistently observed that addition of either saturated ammonium sulfate or acetone to the 5.5 unit stage did not give clear cut fractions; instead stable colloidal solutions formed, from which proteins could be separated only after the removal of the added reagents by dialysis, etc.

Methods of Estimation—Enzyme activity was estimated as follows: 1 cc. of an enzyme solution, 1 cc. of 0.1 M veronal buffer at pH 8.5, and 1 cc. of 0.25 M cyanide[2] in a test-tube were placed in a water bath maintained at 30° for 10 minutes. 0.5 cc. of 0.1 M fructose diphosphate (also at 30°) was rapidly added and the reaction stopped after 10 minutes by the addition of 5 cc. of 10 per cent trichloroacetic acid. The mixture was then diluted to 10 cc., filtered, and 0.5 cc. aliquots were made strongly alkaline by adding 0.5 cc. of 2 N NaOH. After 10 minutes at room temperature, the alkaline mixture was neutralized and estimated for inorganic phosphate by the Fiske and Subbarow method (9). 0.5 cc. aliquots, which were not exposed to alkali, were run simultaneously to correct for inorganic P blanks.

Iodine oxidations were conducted as follows: To a 0.5 cc. trichloroacetate aliquot were added 1.5 cc. of 10 per cent solution of sodium bicarbonate and 0.05 cc. of 1 N iodine; this was allowed to react for 10 minutes at room temperature. 0.1 cc. of N bisulfite was then added to remove excess iodine and the alkali-labile phosphate was estimated as described above.

Dry weights were determined by adding to tared 15 cc. Pyrex centrifuge tubes known volumes of enzyme preparations. Protein was precipitated by adding 5 cc. of 10 per cent trichloroacetic acid; the mixture was centrifuged and washed two times with 10 cc. of 1 per cent trichloroacetic acid.

---

[2] At the beginning of each experiment, 0.25 M cyanide was freshly adjusted to pH 8.5 by adding 6 N HCl with phenolphthalein as the internal indicator.

The precipitates in the centrifuge tubes were then dried at 80° for 12 hours and weighed. Duplicate runs were carried out in each case.

Substrates—Commercially available hexose diphosphates contained about 15 per cent inorganic phosphate (of the total phosphate). For routine experiments, hexose diphosphate was prepared by treating sodium hexose diphosphate with magnesia mixture. Such preparations were free of inorganic phosphate and contained 84 per cent hexose diphosphate as determined enzymatically. Purer samples of the substrate were obtained for equilibrium and resolution experiments by preparing the acid barium salt by the procedure of Neuberg *et al.* (10). Such preparations were free of inorganic phosphate and were found to contain 98 per cent hexose diphosphate by aldolase analysis. Barium fructose-6-phosphate was prepared by the method of Neuberg *et al.* (10), potassium glucose-1-phosphate by a modified method of Sumner and Somers (11). We are indebted to Dr. W. Z. Hassid for a generous sample of synthetic potassium glucose-6-phosphate.

SUMMARY

An aldolase has been isolated from peas and has been purified some 92 times. Its properties, kinetics, and equilibrium constant have been studied and compared with muscle aldolase. Effects of inhibitors have also been investigated.

BIBLIOGRAPHY

1. Herbert, D., Gordon, H., Subrahmanyan, V., and Green, D. E., *Biochem. J.*, **34**, 1108 (1940).
2. Meyerhof, O., and Junowicz-Kocholaty, R., *J. Biol. Chem.*, **149**, 71 (1943).
3. Taylor, J. F., Green, A. A., and Cori, G. T., *J. Biol. Chem.*, **173**, 591 (1948).
4. Dounce, A. L., and Beyer, G. T., *J. Biol. Chem.*, **173**, 159 (1948).
5. Warburg, O., and Christian, W., *Biochem. Z.*, **314**, 149 (1943).
6. Baba, T., *Biochem. Z.*, **275**, 248 (1943).
7. Allen, R. J. L., *New Phytologist*, **39**, 335 (1940).
8. James, W. O., Heard, C. R. C., and James, G. M., *New Phytologist*, **43**, 62 (1944).
9. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
10. Neuberg, C., Lustig, H., and Rothenberg, M. A., *Arch. Biochem.*, **3**, 33 (1943–44).
11. Sumner, J. B., and Somers, G. F., *Arch. Biochem.*, **4**, 11 (1944).

THE EFFECT OF CRYSTALLINE ADRENAL CORTICAL STEROIDS, DL-THYROXINE, AND EPINEPHRINE ON THE ALKALINE AND ACID PHOSPHATASES AND ARGINASE OF THE LIVER AND KIDNEY OF THE NORMAL ADULT RAT*

By CHARLES D. KOCHAKIAN AND MARY N. BARTLETT

(From the Department of Physiology and Vital Economics, School of Medicine and Dentistry, The University of Rochester, Rochester, New York)

(Received for publication, May 7, 1948)

Aqueous adrenal cortical extract (Upjohn), but not desoxycorticosterone acetate, produces a remarkable increase in the "alkaline" phosphatase of the liver of the adrenalectomized rat (1–3). This effect is apparently related to glyconeogenesis from endogenous protein (4). The extract, however, contains in addition to the C_{11} steroids many other substances including epinephrine[1] (less than 1:800,000), which is able to increase liver glycogen with lactic acid as a precursor. It became important, therefore, to determine whether this enzyme phenomenon was a specific property of C_{11} adrenal cortical steroids and also to obtain information as to in what phase in the glyconeogenic process this enzyme was involved. As a means to these ends, a comparison has been made of the effect of aqueous adrenal cortical extract with a crystalline mixture of hog adrenal cortical steroids (Upjohn's lipoextract),[2] 11-dehydrocorticosterone acetate (Merck, synthetic),[3] and epinephrine. Furthermore, a study with thyroxine has been included because it is known to accelerate glycogenolysis due to an increase in energy demands.

At the same time the arginase activity of the liver was studied in order to obtain further information as to the apparent discrepancy between the increase observed by Fraenkel-Conrat *et al.* (5) and the lack of increase noted in this laboratory (4) after administration of adrenal cortical extracts to adrenalectomized rats.

Procedure

Male rats of the Sprague-Dawley strain were placed in individual cages in an air-conditioned room at 25.5–26.6° and fed 10 gm. per day of a pre-

---

* This investigation was aided by grants from the Josiah Macy, Jr., Foundation.

[1] Personal communication, Dr. D. J. Ingle, The Upjohn Company.

[2] The lipoextract (Research No. 8120) was provided by Dr. M. H. Kuizenga of The Upjohn Company on June 21, 1943, and was kept at room temperature. The experiments in this paper were carried out on February 16, 1948.

[3] The synthetic 11-dehydrocorticosterone acetate was provided by Merck and Company on December 19, 1947, and dissolved at 5 mg. per ml. in sesame oil containing 10 per cent benzyl alcohol.

pared diet composed of casein 16.7, sucrose 61.2, hydrogenated vegetable oil 7.4, yeast (Fleischmann's 2019) 9.2, Cellu flour 1.8, Wesson's salt mixture 3.7 (6), and as a daily supplement 1 drop of cod liver oil and 1 drop of a 34 per cent tocopherol concentrate of wheat germ oil[4] diluted 10-fold with Wesson oil. The rats were kept 3 to 4 weeks on this regimen before the experiments were carried out.

The adrenal cortical hormones were administered according to the procedure of Reinecke and Kendall (7). The epinephrine was injected 1 hour before autopsy as a single 0.05 ml. subcutaneous dose of a 1:1000 commercial preparation of the hydrochloride (Parke, Davis and Company). The DL-thyroxine (Roche-Organon, synthetic) was dissolved in a small amount of 0.02 N sodium hydroxide and then made to 2.5 mg. per ml. with water. The rats lost weight rapidly with the initial dose during the first 3 days; therefore, the amount was reduced to 0.25 mg. per day for the remaining 2 days. The last injection was made 24 hours before autopsy.

At the end of the experiments the animals were anesthetized by the intraperitoneal injection of 0.3 ml. of dial-urethane,[5] and the liver was removed and rapidly weighed on a Roller Smith torsion balance. The left segment of the median lobe was used for the enzyme studies (2, 4). The remainder was placed immediately into 5 ml. of hot 30 per cent potassium hydroxide and analyzed for glycogen by the Good-Kramer-Somogyi technique (8), except that the hydrolyzed glycogen was neutralized to phenolphthalein (9). The recently modified Somogyi method (10) was used to determine the reducing substance and the results are expressed as glucose.

The kidneys were removed, weighed, and the left one used for the enzyme studies.

The nitrogen content of the organs of the rats treated with DL-thyroxine and epinephrine was determined by the micro-Kjeldahl technique on aliquots of the enzyme homogenates.

Results

Adrenal Cortical Hormone (*Table I*)—There was the expected formation of liver glycogen under the stimulation of these hormone preparations. The most effective material in the doses used was the lipoextract, then the aqueous extract, and finally the synthetic 11-dehydrocorticosterone acetate. The "alkaline" phosphatase of the liver was greatly increased and to approximately the same extent for each adrenal cortical preparation. There was no parallelism with the degree of glyconeogenesis.

The arginase activity and also the "acid" phosphatase of the liver were not significantly altered.

---

[4] The wheat germ oil concentrate was provided by Distillation Products, Inc., through the courtesy of Dr. P. L. Harris.

[5] The dial-urethane was provided by Ciba Pharmaceutical Products, Inc.

Epinephrine (*Table II*)—The injection of epinephrine produced within 1 hour a tremendous deposition of glycogen in the liver of rats fasted for 18 hours, which was accompanied by a proportionate decrease in the per cent but not total nitrogen (protein) content.

TABLE I

Effect of C_{11} Adrenal Cortical Steroids on Composition and Enzymes of Livers of Male Rats (250 to 252 gm.)

Each group consisted of four rats.

	Dose	Liver	Glycogen total	"Alkaline" phosphatase*	Arginase
		mg.	*mg.*	*units per gm. per cent†*	*units per gm. per cent†*
Control		6640	22	(3.3)	(11,590)
Adrenal cortical extract‡	8 × 1 ml. per hr.	7440	118	+164	−24
Lipoextract§	8 × 0.5 ml. per hr.	7060	154	+161	−17
11-Dehydrocorticosterone acetate (synthetic)‖	8 × 1 mg. per hr.	6270	46	+171	−11

* No effect on "acid" phosphatase of liver or enzymes of kidney.

† Per cent change from control values which are given in parentheses.

‡ Aqueous, Upjohn.

§ Lipoextract = Upjohn's adrenal cortical extract. 1 ml. is equivalent to 2 mg. of 11-dehydro-17-hydroxycorticosterone..

‖ Dissolved at 5 mg. per ml. in sesame oil containing 10 per cent benzyl alcohol.

TABLE II

Effect of Epinephrine and Thyroxine on Composition and Enzymes of Livers of Male Rats

Each group consisted of five rats.

	Dose	Body weight	Liver	Glycogen total	Nitrogen total		"Alkaline" phosphatase	Arginase
		gm.	*mg.*	*mg.*	*mg.*	*per cent*	*units per gm. per cent**	*units per gm. per cent**
Control		287	8230	120	298	3.63	(3.0)	(11,310)
Epinephrine	0.05 mg.†	286	9510	613	279	2.95	−5	−24
DL-Thyroxine	3 × 0.5 mg. per day 2 × 0.25 mg. per day	288‡	7610	7	284	3.71	−33	+3

* Per cent change from controls which are given in parentheses.

† The autopsy was performed 1 hour after injection.

‡ The body weight was 278 gm. at autopsy.

The arginase and "alkaline" phosphatase were not significantly altered.

DL-*Thyroxine* (*Table II*)—The animals injected with thyroxine lost 10 gm. of their body weight with a slight but not significant increase in urinary nitrogen. The liver glycogen was extremely low but the nitrogen (protein) content was comparable with that of the control rats.

The "alkaline" phosphatase was somewhat decreased, but the arginase was not affected.

The weight of the kidney as was expected (*cf.* (11)) increased 22 per cent. Part of this increase was due probably to fat deposition (12). The kidneys were putty-colored. It is of interest that the kidneys increased in size while the rats were on a constant food intake. In previous observations (11) the rats were fed *ad libitum.*

In all of the above experiments there was no change in the "acid" phosphatase activity of the liver or any of the enzymes of the kidney.

DISCUSSION

The similar increases in "alkaline" phosphatase of the liver of the rat after administration of aqueous adrenal cortical extract,[6] lipoextract, and synthetic 11-dehydrocorticosterone acetate indicate that this phenomenon is a property of the S hormones of the adrenal cortex.

The inability of either epinephrine or thyroxine to influence the level of activity of this enzyme in the liver in spite of their marked glycogenic and glycogenolytic properties provides indirect evidence that the enzyme may be concerned with the endogenous protein or amino acid phase of glyconeogenesis. This hypothesis gains further support from the fact that neither a high protein nor a high carbohydrate diet will produce comparable increases in the "alkaline" phosphatase of the liver (3).[7]

The inability of any of the C_{11} steroid preparations to change significantly the arginase level of the liver of the normal rat supports the results obtained in the adrenalectomized rat (3, 4). These data, therefore, provide further evidence that a change in liver arginase level is not essential for glyconeogenesis from protein under the stimulation of the C_{11} steroids as suggested by Fraenkel-Conrat *et al.* (5, 13). Therefore, the decrease in the level of this enzyme after adrenalectomy (5, 4, 14) and hypophysectomy (13)[7] must be due to some other factor or group of factors (3, 4). It is of immediate interest that the decrease in liver arginase after hypophysectomy occurs in spite of an enhanced protein catabolism.[7] Furthermore, intense glycosuria and glyconeogenesis during alloxan diabetes are not accompanied by any change in the arginase activity of the rat liver.[7]

---

[6] The addition of aqueous adrenal cortical extract to the homogenate of normal rat liver does not produce an increase in the "alkaline" phosphatase activity (preliminary experiments).

[7] Unpublished.

The failure of thyroxine to affect liver arginase is in agreement with the negative results obtained by Lightbody and Kleinman (15). It is of interest that Fraenkel-Conrat *et al.* (13) found that the thyrotropic hormone was ineffective in short term experiments but produced a small decrease in hypophysectomized rats after 10 days of treatment. Similar results were obtained with thyroxine.

The failure of any of the C_{11} steroids to influence the enzymes of the kidney of the normal rat is not surprising. The small changes observed in the adrenalectomized rat (2, 4, 14) were in all probability restorative in nature.

SUMMARY

Aqueous (beef) adrenal cortical extract, lipoextract (hog adrenals), and 11-dehydrocorticosterone acetate produced very marked increases in the "alkaline" (pH 9.8) phosphatase of the liver of fasted rats when injected eight times at hourly intervals. The increase in enzyme activity did not parallel the degree of glyconeogenesis. Thyroxine produced a marked depletion of liver glycogen and a decrease in the enzyme. Epinephrine produced a tremendous deposition of liver glycogen but did not affect the activity of the enzyme.

In none of the above treatments were the activities of the arginase and "acid" (pH 5.4) phosphatase of the liver or the enzymes of the kidney altered.

BIBLIOGRAPHY

1. Kochakian, C. D., and Vail, V. N., *J. Biol. Chem.*, **156**, 779 (1944).
2. Vail, V. N., and Kochakian, C. D., *Am. J. Physiol.*, **150**, 580 (1947).
3. Kochakian, C. D., in Recent progress in hormone research; Proceedings of the Laurentian hormone conference, New York, **1**, 277 (1947).
4. Kochakian, C. D., and Vail, V. N., *J. Biol. Chem.*, **169**, 1 (1947).
5. Fraenkel-Conrat, H., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.*, **147**, 99 (1943).
6. Wesson, F., *Science*, **75**, 339 (1932).
7. Reinecke, R. M., and Kendall, E. C., *Endocrinology*, **31**, 573 (1942).
8. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, **100**, 485 (1933).
9. Forbes, W. H., Josiah Macy, Jr., Foundation Conference on metabolic aspects of convalescence, 10th meeting, New York, 157 (1943).
10. Somogyi, M., *J. Biol. Chem.*, **160**, 61 (1945).
11. MacKay, E. M., and MacKay, L. L., *J. Nutr.*, **4**, 33 (1931).
12. Best, C. H., and Campbell, J., *J. Physiol.*, **92**, 91 (1938).
13. Fraenkel-Conrat, H., Simpson, M. E., and Evans, H. M., *Am. J. Physiol.*, **138**, 439 (1943).
14. Folley, S. J., and Greenbaum, A. L., *Biochem. J.*, **40**, 46 (1946).
15. Lightbody, H. D., and Kleinman, A., *J. Biol. Chem.*, **129**, 71 (1939); *Proc. Soc. Exp. Biol. and Med.*, **45**, 25 (1940).

STUDIES ON THE METABOLISM OF RADIOACTIVE NICOTINIC ACID AND NICOTINAMIDE IN MICE*

By LLOYD J. ROTH, EDGAR LEIFER, JOHN R. HOGNESS, and WRIGHT H. LANGHAM

(From the Los Alamos Scientific Laboratory of the University of California, Los Alamos, New Mexico)

(Received for publication, May 21, 1948)

The use of isotopes as tracers, both stable and radioactive, has become a well established technique for the study of intermediate metabolism of many compounds of biological importance. Recently the synthesis of radioactive nicotinic acid and nicotinamide (with C^{14} in the carboxyl group) has been reported by Murray, Foreman, and Langham (1).

In this paper the gross metabolism of radioactive nicotinic acid and nicotinamide in mice is discussed. These results were obtained by determining the amount of radioactivity appearing in the exhaled air, urine, feces, and tissues as a function of time, following their administration by intraperitoneal injection.

EXPERIMENTAL

The apparatus which was used is shown in Fig. 1. Three mice were put into each of several cages, *G*, on glass screen, *K*; air (dried and freed of CO_2 by passage through drierite and ascarite) was drawn into the system through *I* at a rate of approximately 500 ml. per minute as determined by a flow meter. The exhaled CO_2 was collected in a train of three towers containing 20 per cent CO_2-free NaOH. Urine was collected in *A* under toluene. Feces were collected in *B*. In order to minimize the contamination of urine and feces with food, *D* was inserted to trap most of the powdered food particles. A single stick of mouse food $\frac{1}{2} \times 4$ inches was inserted into *E* on elevation *F*, which allowed animals to eat but prevented them from gnawing off any large particles and bringing them into the cage. Water was supplied through *H*. Funnel *C* gave good separation of feces and urine, each relatively uncontaminated with food.

On Day 1 of the experiment 0.7 mg. of radionicotinic acid (50,000 counts per second) was injected intraperitoneally into each of nine mice (Strain CF-1) which were then immediately put into metabolism cages (Fig. 1).

---

* This document is based on work performed under contract No. W-7405-eng-36 for the Atomic Energy Project, and the information covered therein will appear in Division V of the National nuclear energy series as part of the contribution of the Los Alamos Laboratory.

Presented at the 113th meeting of the American Chemical Society.

A second set of nine animals was injected with 0.7 mg. each (50,000 counts per second) of radionicotinamide and similarly treated. The β radiation from these injections was approximately 1 roentgen equivalent per day for the 1st day and much less on succeeding days because of the high excretion rate. Urine, feces, and CO_2 were collected at various time intervals and C^{14} activity determined. All measurements of radioactivity were made with the assembly described by Dauben *et al.* (2).

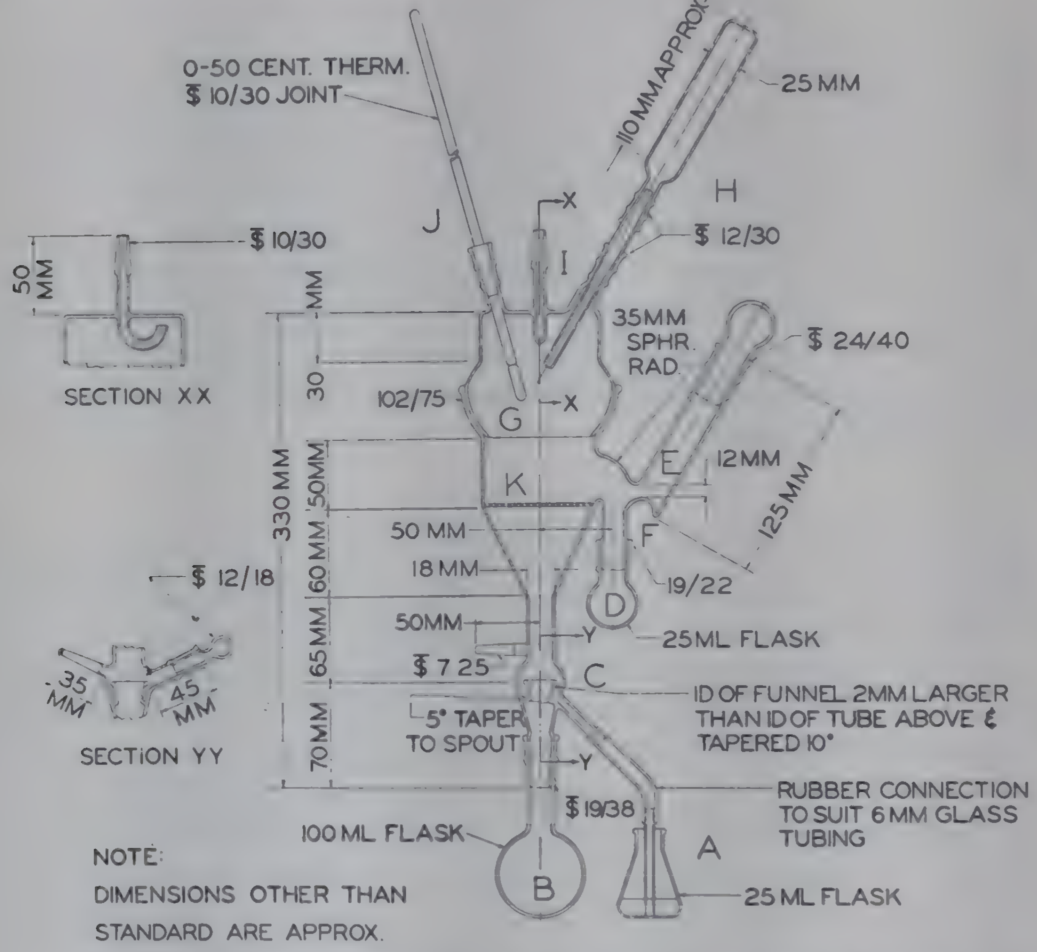

0-50 CENT. THERM.
$ 10/30 JOINT
110 MM APPROX.
25 MM
H
J
X
I
$ 12/30
$ 10/30
50 MM
MM
SECTION XX
35 MM SPHR. RAD.
$ 24/40
30
102/75
G
X
E
12 MM
125 MM
K
330 MM
50 MM
50 MM
F
60 MM
18 MM
19/22
D
$ 12/18
65 MM
50 MM
Y
25 ML FLASK
$ 7 25
C
ID OF FUNNEL 2MM LARGER THAN ID OF TUBE ABOVE & TAPERED 10°
35 MM
45 MM
5° TAPER TO SPOUT
SECTION YY
70 MM
Y
RUBBER CONNECTION TO SUIT 6 MM GLASS TUBING
$ 19/38
100 ML FLASK
A
NOTE: DIMENSIONS OTHER THAN STANDARD ARE APPROX.
B
25 ML FLASK

FIG. 1. All glass metabolism cage for collecting urine, feces, and CO_2

The Geiger-Müller tube used had a thin mica window, 5.4 cm. in diameter and 1.7 mg. per sq. cm. thick. The tube was filled to atmospheric pressure with helium saturated with alcohol at 3°.

Urine samples were collected, diluted to 25 ml., and an aliquot plated directly on oxidized copper disks. The measured activity was corrected for mass absorption with calibration curves developed by preparing plates of increasing amounts of biological fluids containing a constant amount of a water solution of radioactive nicotinic acid.[1]

---

[1] Hogness, J. R., Roth, L. J., Leifer, E., and Langham, W., *J. Am. Chem. Soc.*, in press.

Exhaled carbon dioxide was collected in 20 per cent NaOH and converted to $BaCO_3$. The $BaCO_3$ was precipitated onto oxidized copper disks, counted, and the results corrected for absorption by the method of Yankwich *et al.* (3).

The feces were dried in an oven at 96°, pulverized, and an aliquot extracted with water in a micro Soxhlet apparatus. More than 95 per cent of the C^{14} activity was removed in this manner. An aliquot of the water solution was plated directly and counted.

The data presented for urine, feces, and CO_2 are an average for nine mice.

A second series of mice was injected with similar doses of radionicotinic acid and radionicotinamide. The animals were sacrificed at various time intervals for purposes of tissue analysis. All tissues except blood were dried in an oven at 96°; they were then burned by standard procedures and the CO_2 formed in the combustion was absorbed in 10 per cent CO_2-free NaOH, precipitated as $BaCO_3$, and plated onto copper disks. Radioactivity was determined as above.

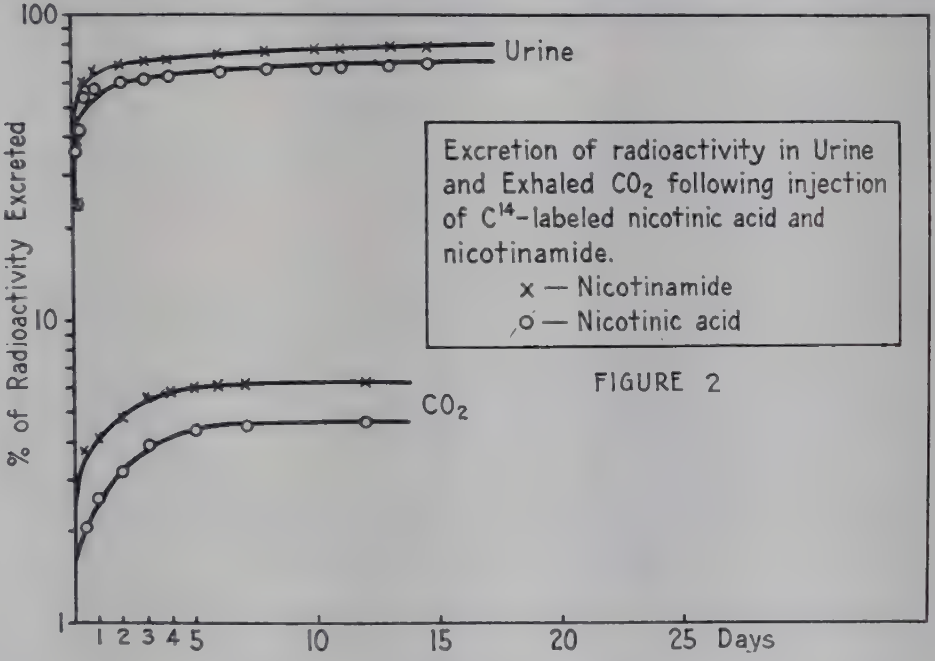


FIG. 2

Blood samples were heparinized and centrifuged. The plasma was separated from the cells and the cells laked with distilled water. Radioactivity in plasma and laked cells was determined by plating the biological fluid directly on copper disks. Corrections for absorption were made according to specially developed absorption curves.

DISCUSSION

Fig. 2 shows the excretion of radioactivity in the urine and CO_2 for nicotinic acid and nicotinamide. Each point represents an average value for the nine animals studied in each group. On Day 1 there is a large excretion of radioactivity in both urine and CO_2. An initial injection of 0.7 mg. of nicotinic acid, which exceeds the normal requirement, results in a large excretion of radioactivity in the first 24 hours. The decrease in the slope of the urine curves which occurs after 24 hours is presumably due to the incorporation of the nicotinic acid and nicotinamide into the metabolic pool of the animal, whereas the initial rapid excretion may be unmetabolized injected material. On the other hand, the rapid initial excretion of CO_2 represents normal metabolism or detoxification. After the first 48 hours, when the nicotinic acid and nicotinamide are presumably in the form of coenzyme in the various tissues, a comparison of the ratio of radioactivity found in the CO_2 and urine would indicate that from 15 to 20 per cent of the fixed nicotinic acid or nicotinamide is eliminated as CO_2. If one assumes that the radioactive CO_2 found in the first 48 hours is due only to the normal metabolic processes, it can be concluded that the total amount of fixed nicotinic acid or nicotinamide is approximately 40 per cent of the injected dose.

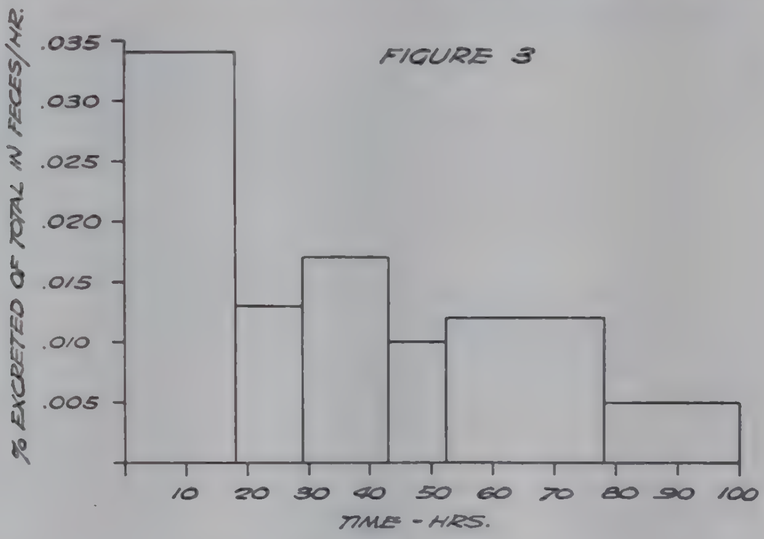


Fig. 3

It is to be noted that the urine curves are parallel, as are the CO_2 curves, indicating that nicotinic acid and nicotinamide enter into the same metabolic system. This is confirmed by the results obtained on individual tissue analysis.

Work on the identification of the various metabolic products in the urine is in progress.

Fig. 3 shows the radioactivity obtained by extracting the feces. The data are plotted as a bar graph to emphasize the irregularities in excretion. Because of the low activity in the feces and the likelihood of contamination

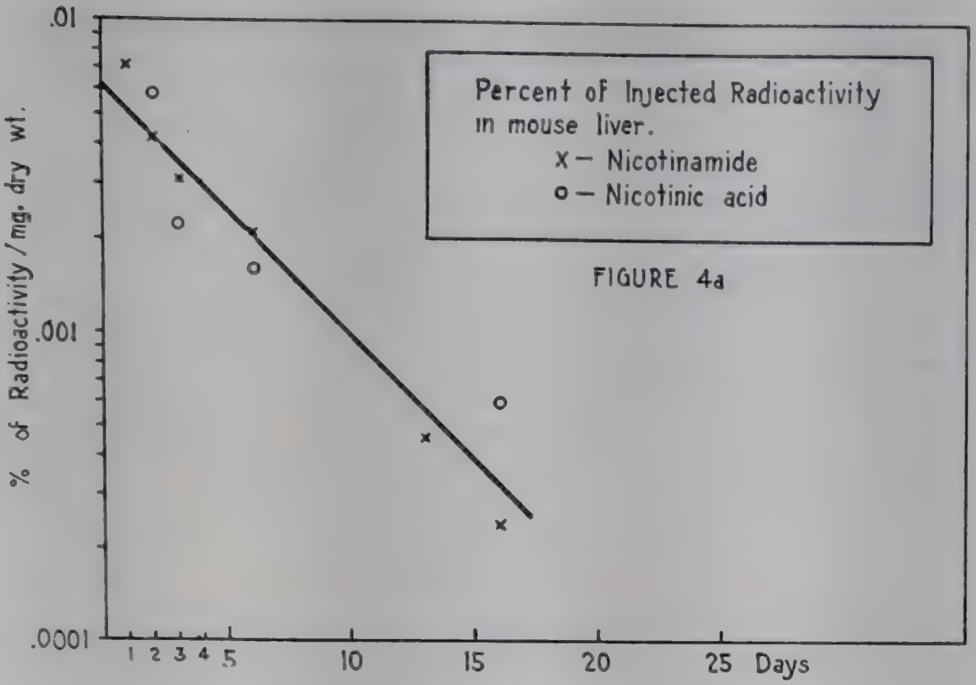


FIG. 4, *a*

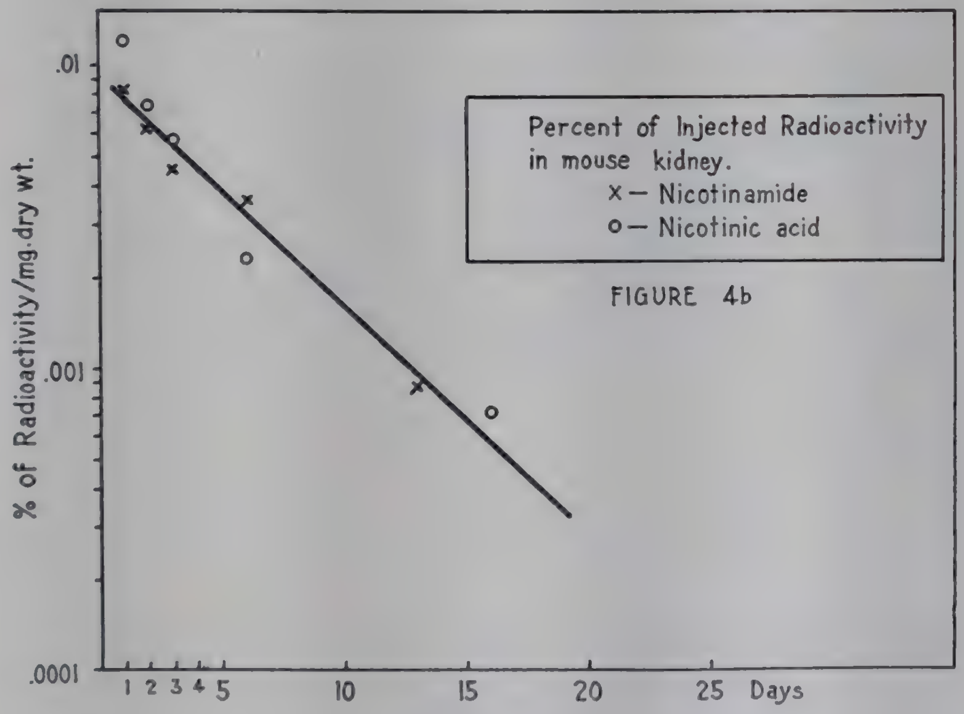


FIG. 4, *b*

from the much more active urine, it is felt that little significance can be attached to these data. If any fecal excretion of metabolites occurs, it is quite low.

The activity in the various tissues resulting from uptake of the injected

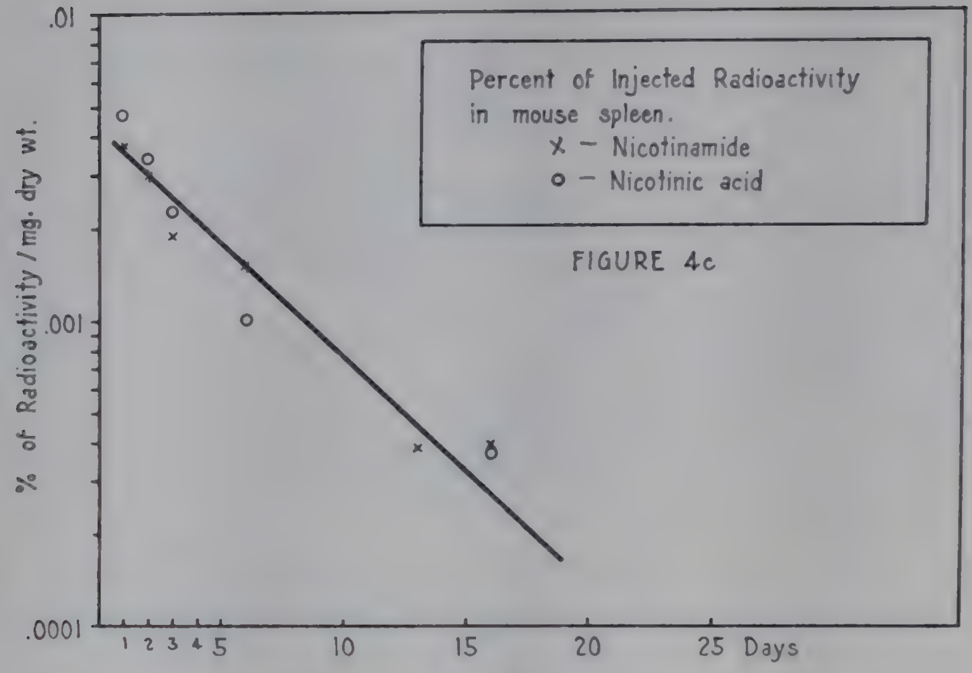


FIG. 4, *c*

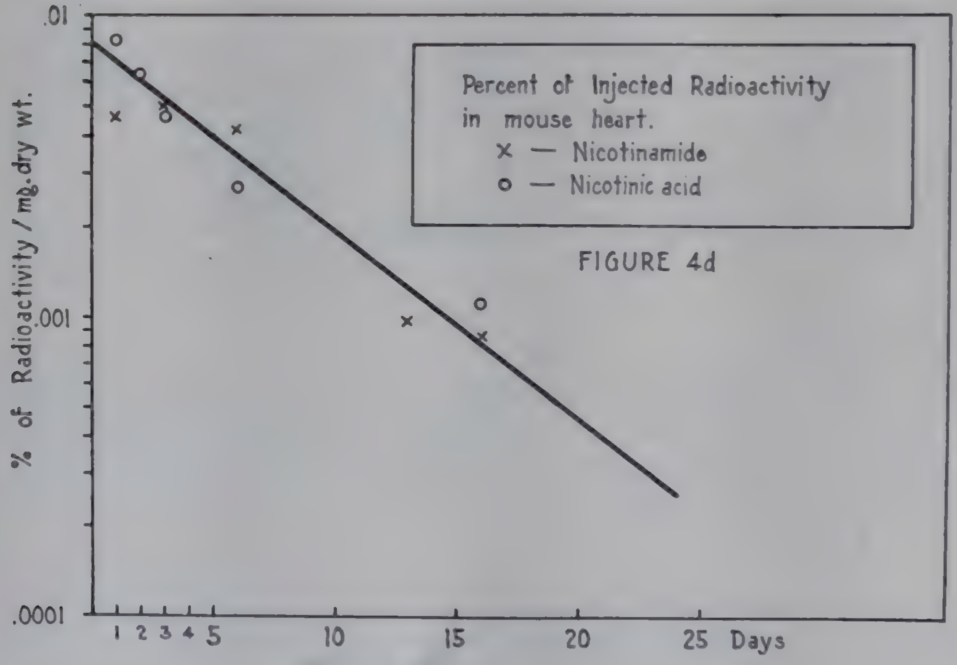


FIG. 4, *d*

nicotinic acid and nicotinamide was determined at increasing time intervals by sacrificing the animals and preparing plates for counting as described above. Fig. 4, *a* through *h*, shows these data on a semilog plot.

The results show that the uptake of radioactive nicotinic acid or nicotinamide varies with the different organs and is highest in kidney and lowest in erythrocytes. No radioactivity was found in the plasma after the first

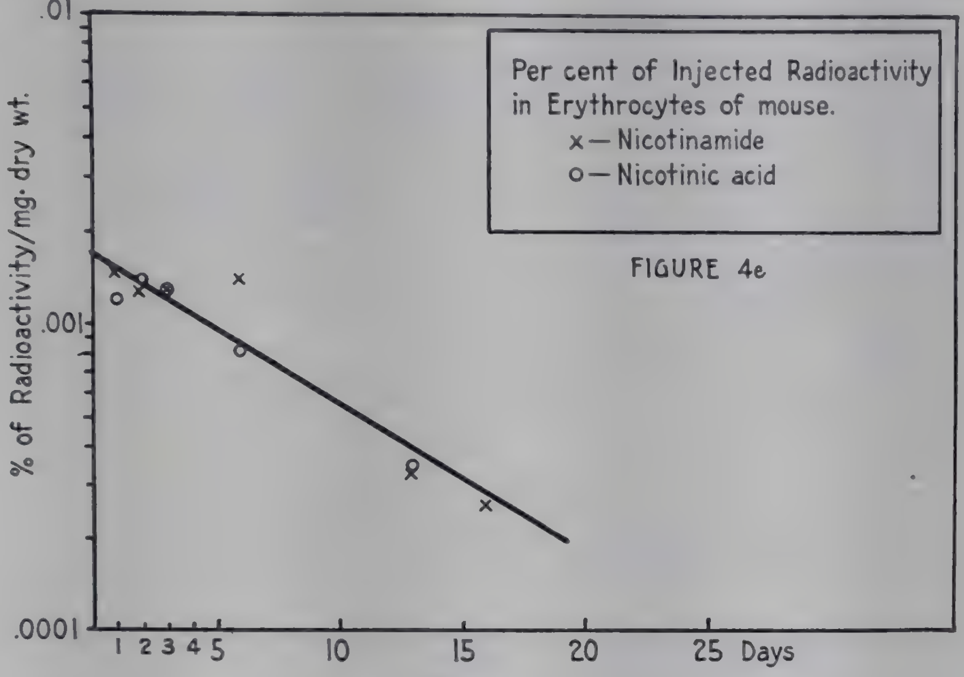


FIG. 4, *e*

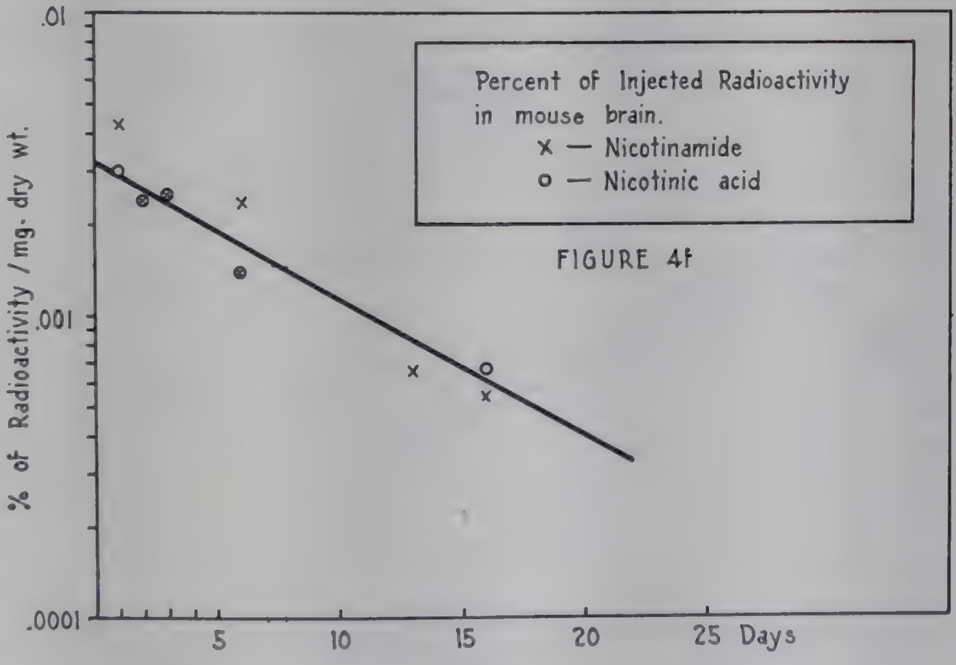


FIG. 4, *f*

24 hours. The excretion half times vary also with the different organs and are about 4 days in liver, kidney, and spleen, 5 days in cardiac muscle and

erythrocytes, and 8 days in brain, sternum, and skeletal muscle. Presumably the major portion of the radioactivity determined was in the form of

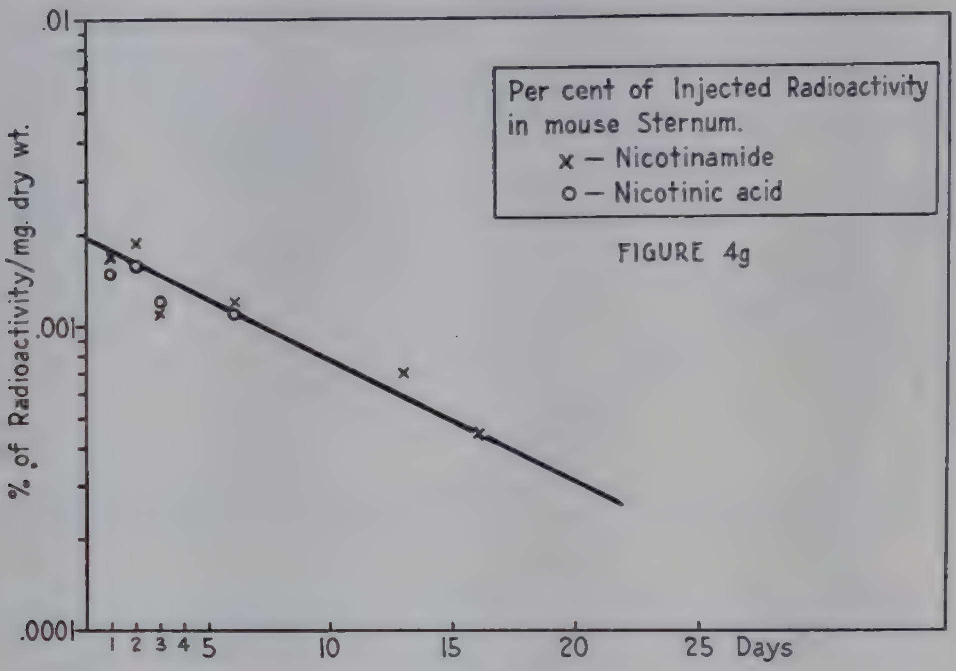


Fig. 4, *g*

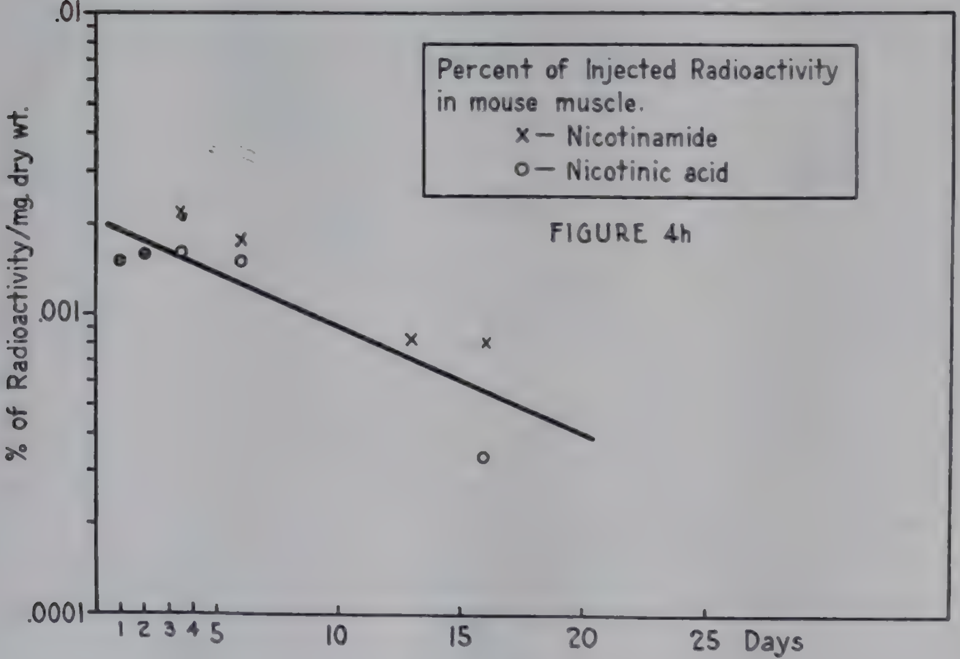


Fig. 4, *h*

coenzyme, as it has been shown repeatedly that nicotinic acid and nicotinamide administered *in vivo* result in an increase in tissue coenzyme.

SUMMARY

1. Gross metabolism studies on nicotinic acid and nicotinamide with use of compounds tagged with C^{14} in the carboxyl group are presented.

2. It is shown that of the radioactivity fixed in the tissues about 15 per cent of the C^{14} in the carboxyl group appears as exhaled CO_2. Presumably this occurs as a result of decarboxylation, but there may also be ring rupture followed by decarboxylation. In any case, the metabolites which have been isolated to date have all contained a carboxyl group in the 3 position of the pyridine ring. The fact that nicotinic acid and nicotinamide are decarboxylated should stimulate the search for additional metabolic products.

3. The gross metabolism of nicotinic acid and nicotinamide is identical in the mouse.

BIBLIOGRAPHY

1. Murray, A., Foreman, W. W., and Langham, W., *Science*, **106**, 277 (1947).
2. Dauben, W. G., Reid, J. C., and Yankwich, P., *Ind. and Eng. Chem., Anal. Ed.*, **19**, 824 (1947).
3. Yankwich, P. E., Norris, T. H., and Huston, J., *Ind. and Eng. Chem., Anal. Ed.*, **19**, 439 (1947).

INTRACELLULAR DISTRIBUTION OF ENZYMES

III. THE OXIDATION OF OCTANOIC ACID BY RAT LIVER FRACTIONS*

By WALTER C. SCHNEIDER

(*From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison*)

(Received for publication, June 19, 1948)

Insoluble residues prepared by low speed centrifugation of tissue homogenates made in isotonic saline have recently been used to study the enzyme systems for the oxidation of fatty acids (6–9, 11), the oxidation of the acids of the Krebs tricarboxylic acid cycle (3), oxidative phosphorylation (10), *p*-aminohippuric acid synthesis (2), and citrulline synthesis (1). Our own studies on the differential centrifugation of rat tissue homogenates have shown that isotonic saline produces extensive agglutination of the mitochondria with the result that the fraction obtained at low speed was a mixture of all of the nuclei and 40 to 80 per cent of the mitochondria of the homogenate in addition to any cells that had not been broken during the homogenization (5, 14). It is, therefore, of interest to determine whether the enzymatic reactions mentioned above are associated with the nuclei, the mitochondria, or the unbroken cells, or whether a combination of two or more fractions is necessary for enzymatic activity. Such a study was made possible by the finding that agglutination was greatly decreased when the homogenates were made in isotonic or hypertonic sucrose solutions (5). Thus a more efficient separation of nuclei and mitochondria was possible in the sucrose homogenates than had hitherto been possible in the saline homogenates.

The present report describes the study of the oxidation of octanoic acid by fractions obtained from rat liver and shows that the oxidation of this fatty acid is associated mainly with the mitochondria.[1]

Materials and Methods

Preparation of Livers—Stock rats originally of the Sprague-Dawley strain obtained from the Holtzman Company, Madison, were fasted overnight to eliminate glycogen from the liver. The rats were killed by decapitation and the livers were removed and chilled in ice-cold isotonic KCl. After

---

* This work was aided by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

[1] This finding confirms the preliminary report of Kennedy and Lehninger (6). No comparison is possible between their results and those obtained in the present work because the tissue concentration used by these authors was not stated.

cooling, the livers were blotted, weighed, and homogenized (12) in ice-cold isotonic sucrose (8.5 gm. of sucrose per 100 ml. of solution). Isotonic sucrose was used rather than the hypertonic sucrose necessary to maintain the morphological integrity of the mitochondria (5), because it was found in preliminary experiments that preparations made in the hypertonic solutions had a much lower rate of fatty acid oxidation than did preparations made in isotonic sucrose. The latter gave activities in the same range as those reported by Potter (11).

Differential Centrifugation of Rat Liver Homogenate[2]—10 ml. of homogenate (1 gm. of rat liver per 10 ml. of homogenate) were centrifuged for 10 minutes at 600*g* to sediment the nuclei. The supernatant was removed and the sediment was washed twice by resuspension in 2.5 ml. portions of isotonic sucrose and recentrifugation at 600*g* for 10 minutes. The washed sediment was made up to 2.5 ml. with isotonic sucrose and labeled the nuclear fraction, N_W. The supernatant and washings from the nuclear fraction were combined and centrifuged 10 minutes at 8500*g* to sediment the mitochondria. The mitochondria were washed twice by resuspension in 2.5 ml. of isotonic sucrose and recentrifugation at 8500*g* for 10 minutes. The washed mitochondria were made up to 2.5 ml. with isotonic sucrose and labeled the mitochondrial fraction, M_W. The supernatant and washings from the mitochondrial fraction were combined and centrifuged for 60 minutes at 18,000*g* to sediment the submicroscopic particles. The submicroscopic particles were washed once by resuspension in 2.5 ml. of isotonic sucrose and recentrifugation at 18,000*g* for 60 minutes. The washed particles were made up to 2.5 ml. with isotonic sucrose and labeled the submicroscopic particles, P_W. The supernatant and washing from the submicroscopic particles were combined, made up to 20 ml., and labeled the supernatant, S_2.

Measurement of "Octanoxidase"[3] *Activity*—Optimum concentrations of the components of the octanoxidase system were determined by Potter (11) and were those employed in this paper. The measurements of oxygen uptake were made in the Warburg apparatus at 38°. The flasks contained 0.01 M potassium phosphate buffer, pH 7.4, 0.01 M potassium malonate,[4] pH 7.4, 0.0133 M magnesium chloride, 0.001 M adenosine triphosphate (K salt), 0.0000133 M cytochrome *c*, 0.028 M NaCl, 0.001 M potassium octanoate, and 0.8 ml. of liver homogenate or an equivalent amount of each fraction. The final volume was 3.0 ml. Readings of oxygen uptake were made at intervals of 5 minutes after a 6 minute equilibration period. The rate of oxygen uptake was constant for a period of at least 20 minutes and was proportional to the tissue concentration. The oxygen uptakes over the 20 minute period were averaged.

---

[2] All of the centrifugations were made at 0° in the International refrigerated centrifuge PR-1. The centrifugations at 600*g* were made with the horizontal yoke No. 269 and those at 8500*g* and 18000*g* were made with the multispeed attachment and the No. 295 conical head. The centrifugal forces designated refer to the centers of the tubes employed.

[3] The term "octanoxidase" will be used to refer to that system of enzymes involved in the oxidation of octanoic acid.

[4] It has been claimed that malonate produces varying degrees of inhibition of octanoxidase activity depending upon the strain of rat employed (9). Since we have

Analytical Methods—Desoxypentosenucleic acid (DNA) and pentosenucleic acid (PNA) were extracted with hot trichloroacetic acid and were estimated by means of colorimetric reactions (13). Total nitrogen was determined colorimetrically after acid digestion (16).

Reagents—The potassium octanoate was obtained by neutralizing octanoic acid (Eastman) that had been distilled *in vacuo* in an all-glass apparatus. Adenosine triphosphate (Ba salt) was generously supplied by Dr. G. A. LePage and was prepared as previously described (16).

Results

Distribution of Nucleic Acids and Total Nitrogen—The distribution of nucleic acids and total nitrogen in the rat liver fractions is given in Table I. The nuclear fraction was found to contain 13.3 per cent of the nitrogen of the homogenate, 13.8 per cent of the PNA, and 99 per cent of the DNA. The latter result indicated that the nuclei had apparently been quantitatively recovered in this fraction. Microscopical examination of the nuclear fraction showed large numbers of intact nuclei both free and clumped together as well as a few unbroken cells and some free mitochondria, while examination of the other fractions failed to show either nuclei or nuclear fragments. The mitochondria fraction contained 26.9 per cent of the nitrogen of the homogenate and 7.2 per cent of the PNA. The submicroscopic particles on the other hand contained 52.6 per cent of the PNA of the homogenate and only 18.8 per cent of the nitrogen. Thus the PNA concentration in the submicroscopic particles was much higher than in any of the other rat liver fractions (76.7 γ of PNA per mg. of N). The supernatant contained the largest proportion of the nitrogen of the homogenate (41.3 per cent) and a considerable amount of PNA (23.2 per cent of the homogenate). The PNA concentration in the supernatant (15.4 γ per mg. of N) was low, however, indicating that the removal of the submicroscopic particles in the previous step of the centrifugation had

---

not experienced significant inhibition in the presence of malonate (Table III), it may be questioned whether the inhibitions observed (9) may not be related to the use of *water* suspensions of the enzyme or to the presence of malate in their assay system.

effectively removed that portion of the liver homogenate containing the highest concentration of PNA.

Distribution of Octanoxidase Activity—The results of the measurements of octanoxidase activity are presented in Table II. With the exception of the supernatant the fractions were tested separately as well as in all possible combinations. The supernatant was tested only separately. The results show that when tested separately only the mitochondrial fraction possessed octanoxidase activity. The activity of the mitochondrial fraction (67 c.mm. of oxygen absorbed per 10 minutes) was increased to 79 c.mm. per 10 minutes when either the nuclear fraction or the submicroscopic particles

TABLE I

Nucleic Acid and Nitrogen Content of Fractions Isolated from Homogenates of Rat Liver in Isotonic Sucrose

The average values are given in bold-faced type.

The figures in parentheses represent the range of the values in the four experiments reported.

Fraction	Nitrogen		DNA phosphorus*			PNA phosphorus*		
	Total†	Fraction of homogenate	Total†	Fraction of homogenate	Per mg. nitrogen	Total†	Fraction of homogenate	Per mg. nitrogen
	γ	*per cent*	γ	*per cent*	γ	γ	*per cent*	γ
Homogenate	**3018** (2967–3048)	(100)	**27.1** (24.9–28.6)	(100)	9.0	**82.8**	(100)	27.4
Nw	**402** (366–430)	13.3	**26.8** (24.8–28.5)	99.0	66.6	**11.4**	13.8	28.3
Mw	**811** (700–890)	26.9				**6.0**	7.2	7.4
Pw	**568** (500–715)	18.8				**43.5**	52.6	76.7
S_2	**1247** (1174–1330)	41.3				**19.2**	23.2	15.4
Recovery		100.3		99.0			96.8	
No. of determinations	4		4			2		

[*] Phosphorus calculated from pentosenucleic acid and desoxypentosenucleic acid determinations.

[†] Per 100 mg. of fresh liver or its equivalent.

were added and to 85 c.mm. per 10 minutes when both of these fractions were added simultaneously to the mitochondria. The reason for the increased activity observed when these fractions were added to the mitochondrial fraction is not known. An explanation for these results may be possible when all the enzymes involved in the octanoxidase system are known and can be tested separately in the fractionation procedure.

Table II

Oxidation of Potassium Octanoate by Fractions Isolated from Homogenates of Rat Liver in Isotonic Sucrose

The average values are given in bold-faced type and represent the experiments for which the nitrogen and nucleic acid content were reported in Table I.

Fraction	Oxygen uptake*			
	+ Octanoate	− Octanoate	Corrected for endogenous	Fraction of homogenate
	c.mm. per 10 min.	*c.mm. per 10 min.*	*c.mm. per 10 min.*	*per cent*
Homogenate	**107** (100–117)	**24** (8–31)	**83** (78–92)	(100)
Nw	**2.3** (0–9.1)		**2.3**	**2.8**
Mw	**67** (64–70)		**67** (64–70)	**81** (71–87)
Pw	**0** (0)		**0**	**0**
Nw + Mw	**79** (72–86)		**79**	**95**
Nw + Pw	**1.1** (0–4.4)		**1.1**	**1.3**
Mw + Pw	**79** (54–94)		**79**	**95**
Nw + Mw + Pw	**85** (69–94)	**1.1** (0–3.0)	**84** (69–94)	**101** (90–105)
S_2	**5.6** (3.4–8.3)	**8.1** (5.6–11.7)		

* Per 100 mg. of fresh liver or its equivalent.

As pointed out in the first paper of this series, one of the fundamental principles that must be observed in fractionation studies, if the results of the assays are to have any significance, is that each fraction must be assayed for enzyme activity and that the total activity of the fractions must closely approximate the activity of the original homogenate. Some difficulty was encountered in adhering to this principle in the study of the octanoxidase system. It is apparent from Table II that the activity of the homogenate (107 c.mm. per 10 minutes) is considerably greater than the activity of the recombined nuclear, mitochondrial, and submicroscopic particle fractions (85 c.mm.). It was observed, however, that the homogenate had a high endogenous uptake of oxygen (24 c.mm.), while these three fractions either separately or in combination had negligible endogenous oxygen uptake. The supernatant on the other hand showed a considerable endogenous oxygen uptake (8.1 c.mm.). When the endogenous uptake was subtracted from the uptake in the presence of octanoate, the activity of the homogenate (83 c.mm. per 10 minutes) agreed closely with the activity of the combined nuclear, mitochondrial, and submicroscopic particle fractions (84 c.mm. per 10 minutes). Inasmuch as the activity of the three recombined fractions agreed reasonably well with the corrected activity of the homogenate in each of four experiments, it was concluded that this correction was valid even though all of the endogenous uptake was not recovered in the fractions. On the basis of the corrected values for the homogenate the mitochondria possessed an octanoxidase activity that was 81 per cent as great as that of the homogenate. Thus it can be concluded that the major portion of the activity of this enzyme system was associated with the mitochondria.

TABLE III

Effect of Repeated Sedimentation of Mitochondria from Isotonic Sucrose on Their Ability to Oxidize Octanoate and on Their Nitrogen Content

Each figure represents the average of duplicate measurements.

Mitochondria preparation	Oxygen uptake*	Nitrogen*
	c.mm. per 10 min.	γ
M (sedimented once)	56.6 (58.3)	780
M_1 (" twice)	52.5 (57.5)	723
M_2 (" 3 times)	54.8 (59.5)	723
M_3 (" 4 times)	55.6 (54.5)	726

* Per the equivalent of 100 mg. of fresh liver. The figures in parentheses represent single measurements of the octanoxidase activities when malonate was omitted from the assay system.

Effect of Repeated Sedimentation on Octanoxidase Activity of Rat Liver Mitochondria—Since the results described in the preceding paragraph indicated that the major portion of the octanoxidase activity of rat liver homogenates was associated with the mitochondria, it was of interest to study the effect of repeated sedimentation of the mitochondria from isotonic sucrose on their octanoxidase activity. The results are presented in Table III. The data show that the total octanoxidase activity remained essentially constant after as many as four sedimentations. The nitrogen content of the mitochondria fraction decreased from 780 γ in the first sedimentation to 723 γ after the second sedimentation. Further sedimentation did not alter the nitrogen content appreciably. The loss of nitrogen that occurred during the second sedimentation was probably due to the removal of soluble material or of particles smaller than the mitochondria.

DISCUSSION

The oxidation of fatty acids by rat liver homogenates is believed to involve two major types of reactions: (1) oxidation of the fatty acid to acetoacetic acid by successive oxidative removal of 2-carbon fragments and their subsequent recondensation, or (2) oxidation of the fatty acid to CO_2 and H_2O by condensation of the 2-carbon units with oxalacetic acid to form acids of the Krebs tricarboxylic acid cycle (8). Lehninger (8) has presented data to show that octanoic acid is quantitatively oxidized in the presence of adenosine triphosphate and magnesium ions to acetoacetic acid by the insoluble material obtained by low speed centrifugation of rat liver homogenates and Potter (11) has presented confirmatory evidence in support of this data. Since the measurements of octanoxidase activity in this paper were made under conditions strictly comparable to those of these authors, it may be concluded that the oxidation of octanoic acid to acetoacetic acid is associated almost exclusively with the mitochondria of the rat liver homogenate (Table II). With respect to the localization of the enzymes involved in the further oxidation of fatty acid (the enzymes of the Krebs cycle), it may be stated that several of the individual enzymes have already been demonstrated to be almost exclusively associated with the large granules or mitochondria of rat liver. Thus the enzymes succinic dehydrogenase, cytochrome oxidase, and cytochrome *c* have been found to be associated with these particulate components of the liver cell (4, 5, 14, 15). The assay of the oxalacetic acid-oxidase system, which appears to be an over-all measure of the enzymes of the Krebs cycle, has also been made on rat liver fractions. A large fraction of the oxalacetic acid oxidase activity of the homogenate was recovered in the mitochondrial fraction. The results of these studies will be described in a subsequent paper.

The demonstration that the octanoxidase activity of rat liver homogenates was associated exclusively with the mitochondria and not with the nuclei would have been impossible if a cytochemical search had not been instituted for a medium in which cells could be disrupted without concomitant agglutination of the mitochondria. Fortunately such a medium was discovered in the non-electrolyte sucrose (5). In the absence of such a study, the biochemist might have been tempted to conclude that oxidation of fatty acids by the insoluble material obtained from tissue homogenates was associated with the nuclei, inasmuch as the size and the physical and chemical properties of the latter overshadow those of the mitochondria.

One instance can already be cited in which an enzymatic function has been assigned to the nuclei present in such a mixed preparation (1). The present report shows that the oxidation of octanoic acid does not require nuclei and demonstrates how the more exact localization of the other enzymatic activities associated with insoluble tissue residues (1–3, 10) may be determined.

SUMMARY

1. Rat liver homogenates in isotonic sucrose were separated by differential centrifugation into nuclear, mitochondrial, submicroscopic particle and supernatant fractions.

2. The distribution of pentosenucleic acid (PNA) and desoxypentosenucleic acid (DNA) was determined in the fractions. The entire DNA of the homogenate was recovered in the nuclear fraction. PNA was found in all of the fractions, but in the submicroscopic particle fraction the PNA concentration per mg. of nitrogen was 2.8 times as great as in the homogenate and 52.6 per cent of the PNA of the homogenate was recovered in this fraction.

3. The distribution of the activity of the enzyme system oxidizing octanoic acid was also studied. It was found that the major portion of the activity of this enzyme system was recovered in the mitochondria fraction.

BIBLIOGRAPHY

1. Cohen, P. P., and Hayano, M. J., *J. Biol. Chem.*, **172**, 405 (1948).
2. Cohen, P. P., and McGilvery, R. W., *J. Biol. Chem.*, **171**, 121 (1947).
3. Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, **172**, 389 (1948).
4. Hogeboom, G. H., Claude, A., and Hotchkiss, R. D., *J. Biol. Chem.*, **165**, 615 (1946).
5. Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.*, **172**, 619 (1948).
6. Kennedy, E. P., and Lehninger, A. L., *J. Biol. Chem.*, **172**, 847 (1948).
7. Lehninger, A. L., *J. Biol. Chem.*, **161**, 413, 437 (1945).
8. Lehninger, A. L., *J. Biol. Chem.*, **164**, 291 (1946).
9. Lehninger, A. L., and Kennedy, E. P., *J. Biol. Chem.*, **173**, 753 (1948).
10. Loomis, W. F., and Lipmann, F., *J. Biol. Chem.*, **173**, 807 (1948).
11. Potter, V. R., *J. Biol. Chem.*, **163**, 437 (1946).
12. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
13. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).
14. Schneider, W. C., *J. Biol. Chem.*, **165**, 585 (1946).
15. Schneider, W. C., Claude, A., and Hogeboom, G. H., *J. Biol. Chem.*, **172**, 451 (1948).
16. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis (1945).

ON HEPARIN MONOSULFURIC ACID*

BY J. ERIK JORPES AND SVEN GARDELL

(From the Chemistry Department (Department II) of the Caroline Institute and the Insulin Laboratory of the Vitrum Company, Stockholm, Sweden)

(Received for publication, May 20, 1948)

The chemical nature of pure heparin has attracted much attention. Even before its chemistry was known attempts were made to isolate crystalline derivatives of it (1, 2). When heparin was found to be a polysulfuric acid ester of a polysaccharide, showing a resemblance to chondroitinsulfuric acid (3), serious attempts were made to crystallize its barium salt (4). This salt has, in fact, a crystalline appearance.

Many findings, however, raise considerable doubt regarding its homogeneity. When the barium salt is recrystallized from a 20 per cent acetic acid solution, an inactivation occurs, probably due to depolymerization, without loss of the crystalline appearance (5). The barium salts contain other cations besides barium (6). The sulfur content and the biological activity vary in different preparations, particularly if heparin samples from different animals are compared (7, 8). It seemed to one of us (9) most plausible to consider the heparin as a polysaccharide extensively esterified with sulfuric acid, and the ordinary heparin preparations as inhomogeneous mixtures of mono-, di-, and trisulfuric acids. Brucine salts of heparin fractions with a sulfur content corresponding to that of all these three acids were also isolated (10). All of them showed an anticoagulant effect, which increased within the same animal species with increasing sulfur content. The possibility of obtaining a homogeneous polysaccharide polysulfuric ester seemed therefore to be very remote. In fact, Kuizenga and Spaulding (11), in 1943, separated different fractions with different sulfur contents from a barium salt assumed to be crystalline. As will be reported further on, we have made the same observation.

So far, attention was focused only on the fractions having the highest sulfur content and the strongest anticoagulant activity. The sulfur content of some fractions also reached the figures calculated for a trisulfuric acid, 13.6 per cent for the sodium salt (12) and 10.8 per cent for the barium salt (7, 9, 13). A sodium salt of such a fraction from ox liver with 130 Toronto units per mg. was, moreover, in 1942 selected by the Department of Biological Standards of the National Institute for Medical Research, London, to serve as the provisional international heparin standard.

---

* This investigation was aided by grants from the Knut and Alice Wallenberg Foundation, Stockholm.

The question then arises whether the more soluble fractions of the brucine and barium salts with a lower sulfur content and a weaker anticoagulant activity are chemical entities *per se* or only mixtures of one or several indifferent polysaccharides and the trisulfuric acid ester. We have now analyzed these fractions more closely and found that after removal of at least all the trisulfuric acid they still have an anticoagulant effect and a sulfur content which corresponds in the main fraction to that of the monosulfuric acid of heparin.

Fractionation of Easily Soluble Barium Salts

In the Charles and Scott procedure of 1933 (2) for the large scale extraction of heparin from ox liver and ox lungs, the alkaline extract is acidified with sulfuric acid. Heparin and chondroitinsulfuric acid are thereby precipitated together with the proteins. After complete deproteinization, the heparin is precipitated as a barium salt insoluble in water. The soluble barium salts of the mother liquor are precipitated with alcohol.

Although there is a marked difference in the solubility of the two main fractions of barium salts, a certain amount of heparin tri- and disulfuric esters could nevertheless be assumed to be present in the mother liquor among the easily soluble barium salts, thus explaining their anticoagulant activity. Steps were therefore taken to remove any admixture of ordinary heparin that might be present.

To a 20 per cent aqueous solution of the easily soluble barium salts barium chloride was added to 5 per cent, followed by 0.1 volume of a hot saturated solution of barium hydroxide. After standing for 24 hours in the ice box, the precipitate was removed. It usually comprised 2 per cent of the weight of the easily soluble barium salts. Another fraction of similar size was removed by adding 0.05 volume of alcohol. Both these small fractions contained about 6 to 7 per cent sulfur and about 15 heparin units per mg. of dry substance. The preparations from liver and lungs behaved similarly in these respects. The admixture of trisulfuric acid must consequently have been insignificant, and after removal of these fractions this acid is still less likely to occur as an impurity in the barium salts to be discussed below.

The absence of less soluble barium salts was easily demonstrated by the following procedure.

If half the volume of a saturated solution of barium chloride was added to a neutral 5 per cent solution of the easily soluble barium salts which had been precipitated with 0.1 volume of alcohol (Samples A and B in Table I), no opalescence was seen, not even after standing for 24 hours in the ice box. If the solution contained 0.05 per cent of the sodium salt of heparin with 11.5 per cent sulfur and 110 units per mg. simultaneously, an opalescence was observed on adding the barium chloride. On the following day there was a slight precipitate adhering to the bottom of the test-tube. Twice the amount of heparin, or 0.1 per cent, immediately gave a strong opalescence and on the following day an ordinary precipitate. If heparin had been added to 0.25 per cent, a precipitate formed immediately when the barium chloride solution was added.

The same occurred if protamine, free of sulfate ions, was added to these solutions. 0.5 mg. of protamine base added to 0.5 ml. of a 5 per cent solution of the easily soluble barium salts gave an opalescence. If the solution contained 0.1 or 0.25 per cent of heparin simultaneously, a heavy precipitate formed on adding the protamine.

In these tests a 5 per cent solution of the easily soluble barium salts gave no precipitate with barium chloride, whereas such a low concentration of heparin as 0.05 or 0.1 per cent could easily be demonstrated. The anticoagulant effect of the easily soluble barium salts, 10 to 16 units per mg., would have demanded an admixture of barium salt of ordinary heparin, making up about 10 per cent of their weight. It is therefore evident that these polysaccharide fractions with only one sulfuric acid group for each disaccharide unit also have an anticoagulant effect of their own.

The less soluble barium salts of ordinary heparin thus having been removed, the easily soluble barium salts were precipitated with alcohol from the alkaline solution containing 5 per cent of barium chloride, a first fraction being obtained with 0.1 volume of alcohol and the remainder with 1 volume. This separation into two fractions proved to be very valuable. The barium salt of the lung polysaccharide which precipitated with 0.1 volume of alcohol behaved in quite a different way from the fraction obtained with 1 volume. The former exerted an anticoagulant activity, contained glucosamine, and was dextrorotatory, whereas the latter was biologically inactive and only weakly dextrorotatory. It consisted of chondroitinsulfuric acid.

The amount of soluble barium salts obtained after removal of ordinary heparin is different when one is working with samples from liver and from lung. In the liver it is somewhat larger than the amount of sodium salt of heparin recovered from the insoluble barium salt, the proportions being 1.2:1. With lung the yield is smaller, the soluble barium salts comprising only a third of the weight of the heparin. Furthermore, the easily soluble barium salts from these two sources also differ as to their composition, chondroitinsulfuric acid making up about a third of the samples from lung.

The barium salts thus obtained were reprecipitated twice with alcohol, the second time after neutralization of the solution with hydrochloric acid. Finally they were dissolved in a small volume of water and kept in the cold overnight. Two fractions were thereby separated, Sample A, insoluble, and Sample B, soluble (see Table I), both being analyzed separately.

Before analysis, all the barium salts were once more dissolved in a small volume of water and dialyzed against distilled water in cellophane sacs. The results of the fractionation are given in Table I. For comparison the theoretical figures for the nitrogen, sulfur, and ash content of the barium salt of the heparin monosulfuric acid are given in Table I.

Analysis of Easily Soluble Barium Salts

Sulfur—The sulfur content of the main fraction of the easily soluble barium salts both from lung and liver, which was precipitated with 0.1 volume of alcohol, was about that calculated for a heparin monosulfuric acid (see Table I). Sample B from lung contained 5.42 per cent of sulfur and from liver 5.69 per cent (calculated, 5.38 per cent). Fraction A, which precipitated at a low temperature, had a somewhat higher sulfur content, 6.67 and 6.30 per cent, as was also to be expected for the less soluble fraction. Evidently, it contained an admixture of disulfuric acid of heparin polysaccharide.

TABLE I

Fractionation of Easily Soluble Barium Salts

	Air-dry substance	Dry substance			Heparin in mg. dry substance	$[\alpha]^{20}_{D}$,* dry substance
		Ash	N	S		
	gm.	*per cent*	*per cent*	*per cent*	*units*	*degrees*
Barium salt of heparin monosulfuric acid, $C_{14}H_{19}SO_{14}NBa$, mol. wt. 594.58		39.2	2.36	5.38		
Ox lung. Crude easily soluble Ba salts	1000	33.0		5.25	10–11	
0.1 volume alcohol, Sample A	210	37.8	2.37†	6.67	16	+45.8
0.1 " " " B	127	39.6	2.61	5.42	10	+49.4
1 volume alcohol	230	33.1	2.41	4.44	1.2	+0.62
Ox liver. Crude easily soluble Ba salts	1000	35.2		5.15	18–20	
0.1 volume alcohol, Sample A	14	41.9		6.30	16	
0.1 " " " B	505	39.6	5.43	5.69	18 (?)	
1 volume alcohol	92	35.3	3.90	4.13	7	

* 2 per cent in water.

† 5 per cent of the nitrogen occurred as NH_2 nitrogen.

Amino Sugar—On acid hydrolysis 37 per cent of the calculated amount of amino sugar was isolated as pure crystalline glucosamine hydrochloride. The high yield of glucosamine thus isolated almost excludes any considerable admixture of other amino sugars. Consequently, the amino sugar is glucosamine, as in ordinary heparin (14).

Isolation of Glucosamine—30 gm. of air-dry substance of Sample B (Table I, from lung) were hydrolyzed 7 hours in 500 ml. of 20 per cent hydrochloric acid by heating under a reflux on a metal bath. Hydrochloric acid was removed by repeated evaporations *in vacuo* to almost dryness, the last time after decolorizing with norit. The dry residue was treated with 3 ml. of hot normal hydrochloric acid to which 50 ml. of methyl alcohol were added. The insoluble residue, weighing 3.1 gm., proved to be pure crystalline glucosamine hydrochloride. A 2 per cent solution gave after 2 minutes $[\alpha]_D^{20} = +100°$ and on the following day +71°. From the methyl alcohol another lot of crystals weighing 0.120 gm. was obtained when acetone was added. Total yield, 3.22 gm. of glucosamine hydrochloride or 13.4 per cent of the dry substance of Sample B, Table I. Consequently, the yield of glucosamine hydrochloride was 37 per cent of the theoretical (36.2 per cent).

In another hydrolysis, 1.87 gm. of glucosamine hydrochloride was obtained from 20 gm. of air-dry substance of Sample B, or almost the same yield, this time following hydrolysis in the presence of 10 gm. of stannous chloride, as recommended by Levene.

Exactly the same yield of crystalline glucosamine hydrochloride, 37 per cent of the theoretical, was obtained from 30 gm. of air-dry substance, as in Table I. Consequently, the amino sugar of the heparin monosulfuric acid is glucosamine as is that of the trisulfuric acid.

The well known difficulty of obtaining galactosamine from chondroitinsulfuric acid was also experienced here.

Simultaneously with the above hydrolyses of Samples A and B, 30 gm. of air-dry substance of the last fraction of barium salts from lung, which had been precipitated with 1 volume of alcohol, were hydrolyzed without stannous chloride, and 20 gm. after addition of stannous chloride, when both were submitted to exactly the same procedure as described above. In none of these hydrolyses were any crystals obtained on adding methyl alcohol. On adding acetone in both instances an insoluble sticky mass precipitated, a chondrosine polymer, very familiar to everyone who has attempted to isolate chondrosamine from chondroitinsulfuric acid. This detail, together with the low optical activity and the lack of stronger anticoagulant activity, made it quite evident that this *fraction of the easily soluble barium salts from lung consisted almost exclusively of chondroitinsulfuric acid.* It also behaved like this acid on acid hydrolysis, and against the Schiff reagent after oxidation with periodic acid.

A quantitative analysis for *uronic acid*, with the technique applied by Jorpes and Bergström in 1937 (15), gave 25.0, 30.0, and 30.0 (mean 28.35) per cent (calculated, 32.6 per cent).

An *acetyl determination* according to the method of Lumieux and Purves (16) with chromic acid oxidation gave 7.8, 7.31, and 7.8 (mean 7.5) per cent acetic acid (calculated 10.1 per cent).

Conclusions—The organic skeleton of the polysaccharide with one sulfuric acid group for each disaccharide unit thus consists of 1 molecule of amino sugar, glucosamine, 1 molecule of uronic acid, and 1 molecule of acetic acid.

Because of the anticoagulant activity, 10 to 16 heparin units per mg. of dry substance, and the similarity to heparin, the substance is assumed to be a monosulfuric ester of the heparin polysaccharide.

The fraction of the easily soluble barium salts which precipitated with 1 volume of alcohol consisted, in the preparation from lung, almost exclusively of chondroitinsulfuric acid. It made up a third of the weight of the purified, easily soluble barium salts. It was practically devoid of anticoagulant activity, had a very low dextrorotation, and gave no glucosamine after acid hydrolysis. The corresponding fraction of the easily soluble barium salts from cattle liver, however, showed a similarity to the monosulfuric ester, although it had a somewhat lower sulfur content. It made up only 9 per cent of the weight of the crude, easily soluble barium salts. The difference between these two preparations, from lung and liver respectively, was very clearly demonstrated through their rate of hydrolysis in 7.5 per cent (by volume) sulfuric acid and through their behavior in the fuchsin-sulfurous acid test after periodate oxidation. The preparation from lung behaved in these respects like chondroitinsulfuric acid, and that from liver like monosulfuric ester of heparin.

Acid Hydrolysis for Differentiating Polysaccharides

The anticoagulant activity of heparin is lost when a solution of the barium salt is heated repeatedly to 65° in 20 per cent acetic acid (5), probably owing to a depolymerization. The sulfuric acid is also very easily split off, about 60 per cent of it being hydrolyzed in 5 minutes and all of it in 1 hour on boiling with 2 N hydrochloric acid. The carbohydrate skeleton, however, is fairly resistant to acid hydrolysis, practically no reducing substance being liberated during 60 minutes boiling in 7.5 per cent (by volume) sulfuric acid. This remarkable property of the heparin polysaccharide was observed when the behavior of heparin on acid hydrolysis was compared with that of other similar polysaccharides (15). The chondroitinsulfuric acid and the easily soluble fractions of the brucine salts of heparin hydrolyzed much more easily.

We have found this principle of characterizing a polysaccharide very useful. All the samples of the heparin monosulfuric acid from liver and lung (Samples 1 to 5, Fig. 1) showed the same characteristic rate of hydrolysis.

Heparin monosulfuric acid hydrolyzes more easily than di- and trisulfuric acids but with considerably lower speed than chondroitinsulfuric acid. This indicates a difference in the internal structures of chondroitinsulfuric acid and heparin monosulfuric acid. The same circumstance is furthermore demonstrated by their different response to the Schiff reagent, fuchsinsulfurous acid, after periodate oxidation, the latter giving a strong reaction, while chondroitinsulfuric acid gave practically none (17).

Heparin monosulfuric acid can also be differentiated from hyaluronic acid, which is hydrolyzed with even greater speed than chondroitinsulfuric acid. *Heparin monosulfuric acid is consequently not a monosulfuric ester of hyaluronic acid.*

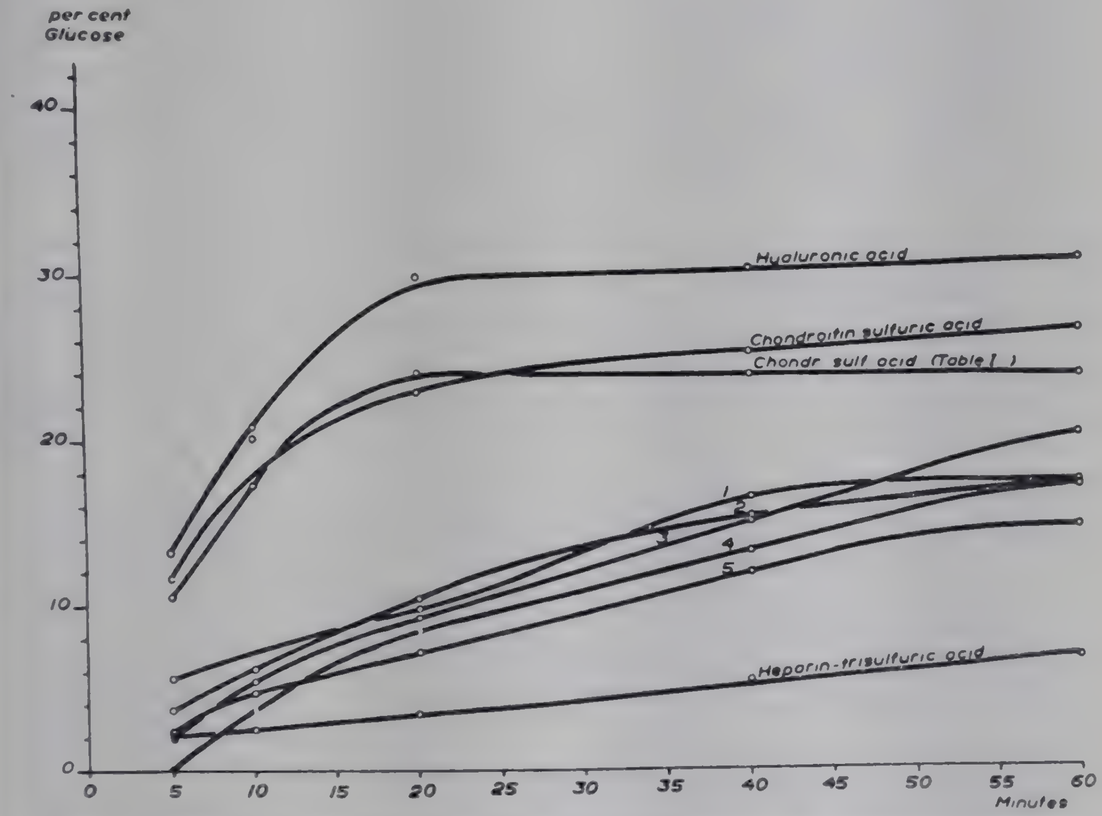


FIG. 1. Reducing substance, calculated as glucose in per cent of the organic material liberated during hydrolysis in 7.5 per cent (by volume) sulfuric acid. The figures on the curves refer to sample numbers.

The most soluble fraction of the easily soluble barium salts of lung heparin behaved in this respect like chondroitinsulfuric acid, whereas that from the liver hydrolyzed like the heparin monosulfuric acid.

Is Heparin Monosulfuric Acid Preformed in Tissues?—The first question which arises is whether the polysaccharide with low sulfur content and weak anticoagulant activity extracted from ox liver and ox lung together with ordinary heparin really occurs as such in the living organisms, or whether it is a degradation product of heparin. The question is by no means easy to answer. Evidently it could result from the action of sulfatases from the heparin already in the living cells. The occurrence of a heparinase has also been discussed by Jaques (8).

This question cannot be answered without further analytical work along the same lines as those applied by Jorpes, Holmgren, and Wilander in 1937 (see Jorpes (9) pp. 57–67), when they demonstrated that there is a very close relationship between the number of mast-cells in the different organs of different animals and their content of heparin.

The next question is whether the monosulfuric acid is formed from heparin through the extractions process. This question has been discussed earlier ((12) p. 209). Tryptic digestion does not break down any heparin. Heparin submitted to the same treatment as the lungs and the liver with an ammoniacal ammonium sulfate solution of pH 9 to 9.4 could be recovered unchanged, although a quantitative yield was not to be expected. The sulfate groups, however, are rather easily hydrolyzed. From heparin trisulfuric acid 33 per cent of the sulfur is split off at 100° in 0.1 N sodium hydroxide during 5 minutes, 36 per cent in 15 minutes, 54 per cent in 1 hour, and 66 per cent in 4 hours. After 24 hours boiling in 5 N sodium hydroxide only 72 per cent is split off. In an alkaline solution one sulfate group is thus very easily removed from the trisulfuric acid, the second more slowly, and the third with the utmost difficulty. The sulfuric acid is, however, in alkaline solution, removed from the monosulfuric acid with about the same speed. The monosulfuric ester can therefore not arise from di- and trisulfuric esters in the course of alkaline extraction.

Also, in a weakly acid solution, the sulfate groups are liberated at about the same speed from the mono- as from the di- and trisulfuric esters.

Against this background the question of the chemical nature of heparin can more easily be discussed. Fractions with the composition of a trisulfuric acid have earlier been isolated and described. Here we describe a monosulfuric ester of a polysaccharide, in many respects resembling heparin. Ordinary heparin which precipitates as an insoluble barium salt from water is neither a trisulfuric acid nor a monosulfuric acid but a mixture of tri- and di- and possibly also some monosulfuric ester. Even a tetrasulfuric ester might conceivably occur, as can be concluded from the following experiment. We obtained from 74 gm. of barium salt, "recrystallized" twice from 10 per cent acetic acid after heating to 60° through repeated dissolving in hot water, a number of fractions which after transformation into sodium salts gave the following yield of sulfur: 15.83 gm. 13.6 per cent, 2.14 gm. 10.88 per cent, 3.36 gm. 8.23 per cent, 6.24 gm. 7.60 per cent, 7.36 gm. 4.48 per cent.

The high yield of the sodium salt with 13.6 per cent sulfur, the theoretical figure for the sodium trisulfuric acid of heparin, makes an admixture of a tetrasulfuric acid in this sample very probable. So far, however, we have no conclusive evidence as to the occurrence of this acid.

DISCUSSION

In discussing the possibilities at hand against the background of the analytical findings, one feels almost inclined not to speak of the different mono-, di-, and trisulfuric esters but to consider the heparin as a polysaccharide more or less esterified with sulfuric acid, in which all the different disaccharide units of one and the same molecular complex need not necessarily be esterified to the same degree. The accumulation of sulfate groups determines the solubility of the barium salts and improves the anticoagulant activity. There are samples with about three sulfate groups for each disaccharide unit, samples with only one group and intermediates, probably a mixture of di- and trisulfuric acid esters. The occurrence of a tetrasulfuric acid of the same polysaccharide is very likely, as is also the occurrence of the sulfur-free heparin polysaccharide itself in the animal body.

SUMMARY

The main fraction of the easily soluble barium salts remaining in the mother liquor when protein-free heparin from ox liver and ox lung is precipitated as an insoluble barium salt has the composition of a heparin monosulfuric acid. It contains equal parts of glucosamine, a uronic acid, acetic acid, and sulfuric acid. It is dextrorotatory, the barium salt having an optical rotation of approximately +50°.

The preparations are practically free of heparin di- and trisulfuric acids and have an anticoagulant activity, in the form of the barium salt, between 10 and 16 provisional international heparin units per mg.

The rate of hydrolysis at 100° in 7.5 per cent (by volume) sulfuric acid distinctly differentiates this polysaccharide from the chondroitinsulfuric acid which hydrolyzes much faster, and from the hyaluronic acid which hydrolyzes even more rapidly.

The yield of this fraction from ox liver almost equals that of heparin, but that from lung makes up only a fifth.

All of the heparin preparations are more or less inhomogeneous. This applies to the strongest ones, with approximately three sulfate groups for each disaccharide unit, as well as to the monosulfuric acid. It is questionable whether any chemically well defined heparin samples can be obtained.

The authors are greatly indebted to Mr. Gunnar Lindén, Apoteksvarucentralen Vitrum, and to Mr. Viktor Mutt for their assistance during the performance of the work.

BIBLIOGRAPHY

1. Fischer, A., and Schmitz, A., *Z. physiol. Chem.*, **216**, 264, 274 (1933).
2. Charles, A. F., and Scott, D. A., *J. Biol. Chem.*, **102**, 425, 437 (1933).

3. Jorpes, J. E., *Biochem. J.*, **29,** 1817 (1935).
4. Charles, A. F., and Scott, D. A., *Biochem. J.*, **30,** 1927 (1936).
5. Wolfrom, M. L., Weisblat, D. I., Karabinos, J. V., McNeely, W. H., and McLean, J., *J. Am. Chem. Soc.*, **65,** 2077 (1943).
6. Charles, A. F., and Todd, A. R., *Biochem. J.*, **34,** 112 (1940).
7. Jaques, L. B., *Science*, **92,** 488 (1940).
8. Jaques, L. B., *J. Biol. Chem.*, **133,** 445 (1940).
9. Jorpes, J. E., Heparin in the treatment of thrombosis, London and New York (1946).
10. Jorpes, J. E., and Bergström, S., *Biochem. J.*, **33,** 47 (1939).
11. Kuizenga, M. H., and Spaulding, L. B., *J. Biol. Chem.*, **148,** 641 (1943).
12. Jorpes, J. E., *Biochem. J.*, **36,** 203 (1942).
13. Jaques, L. B., Waters, E. T., and Charles, A. F., *J. Biol. Chem.*, **144,** 229 (1942).
14. Jorpes, J. E., and Bergström, S., *Z. physiol. Chem.*, **244,** 253 (1936).
15. Jorpes, J. E., and Bergström, S., *J. Biol. Chem.*, **118,** 447 (1937).
16. Lumieux, R. U., and Purves, C. B., *Canad. J. Res.*, *Sect. B*, **25,** 485 (1947).
17. Jorpes, J. E., Werner, B., and Åberg, B., *J. Biol. Chem.*, **176,** 277 (1948).

THE FUCHSIN-SULFUROUS ACID TEST AFTER PERIODATE OXIDATION OF HEPARIN AND ALLIED POLYSACCHARIDES*

By J. ERIK JORPES, BIRGITTA WERNER, and BERTIL ÅBERG

(From the Chemistry Department (Department II) of the Caroline Institute and the Insulin Laboratory of the Vitrum Company, Stockholm, Sweden)

(Received for publication, June 19, 1948)

Oxidation with periodic acid affords a possibility of drawing conclusions regarding the internal structure of some polysaccharides. Since two adjacent hydroxyl groups are required, some compounds such as heparin trisulfuric acid, in which sulfate groups occupy three of the four hydroxyl groups assumed to be present, will not react with periodic acid. Ordinary heparin has therefore been found not to consume periodate (1). It also gives a negative reaction with fuchsin-sulfurous acid after treatment with periodic acid. A fraction of the heparin polysaccharide with a lower sulfur content, the heparin monosulfuric acid described in the preceding paper (2), on the contrary, consumes iodate and gives a strongly positive reaction in the color test.

In applying the color test to different polysaccharides related to heparin it was found that chondroitinsulfuric acid, which has the same elementary composition as heparin monosulfuric acid, gives a negative test. This result was somewhat unexpected. The different behavior of the two related polysaccharides can, however, easily be explained.

Depending on the linkages between the two main components of these mucopolysaccharides, the amino sugar and the uronic acid, the sulfate-free polysaccharide will have two adjacent hydroxyl groups either in both of the components of the disaccharide unit (*cf.* (2) Fig. 1, upper structure), in one (lower structure), or in none of them. When a hydroxyl group becomes esterified with sulfuric acid, one α-glycol structure is eliminated.

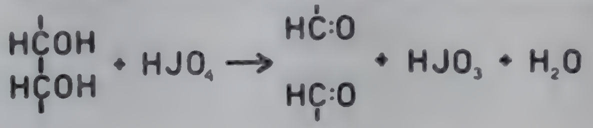
The different behavior of the two related compounds, heparin monosulfuric acid and chondroitinsulfuric acid, to periodic acid consequently discloses a difference in the internal structure of the two polysaccharides. Furthermore, the same was demonstrated when the polysaccharides were submitted to acid hydrolysis in 7.5 per cent (by volume) sulfuric acid (*cf.* (2) lower structure, Fig. 1).

The reaction with periodic acid can also be used for staining polysaccharides in animal and plant tissues, as suggested recently by Hotchkiss

---

* This investigation was aided by grants from the Knut and Alice Wallenberg Foundation, Stockholm.

(3), the reaction products, the aldehydes, giving a red color with fuchsin-sulfurous acid. Since ordinary heparin does not react and heparin monosulfuric acid gives a positive reaction, we applied this color reaction to the tissue mast-cells, the heparinocytes, which are known to produce heparin, hoping to find a positive reaction indicating the presence of the monosulfuric acid in these cells. Actually, the granules of the heparinocytes of the subcutaneous tissue of the young rat readily gave a very intense red color with fuchsin-sulfurous acid after treatment of the tissue slices with periodic acid, but no color without this treatment. The histological technique, however, is not yet fully elaborated and does not allow any final conclusions to be drawn. Many sources of error must first be eliminated. Thus, for example, oxidants such as chromic acid cannot be used for fixation of the material, and the fuchsin itself has a great affinity for the tissues. The question of the occurrence of the heparin monosulfuric acid in the tissues will be studied further by us both chemically and histologically. The resistance of the chondroitinsulfuric acid to periodate could easily be demonstrated histologically. In staining sections from trachea with the Hotchkiss technique, the mucous cells acquired a bright red color from hyaluronic acid, whereas the cartilage remained completely unstained.



$$\begin{matrix}\text{HCOH}\\ \text{HCOH}\end{matrix} + HJO_4 \rightarrow \begin{matrix}\text{HC:O}\\ \\ \text{HC:O}\end{matrix} + HJO_3 + H_2O$$

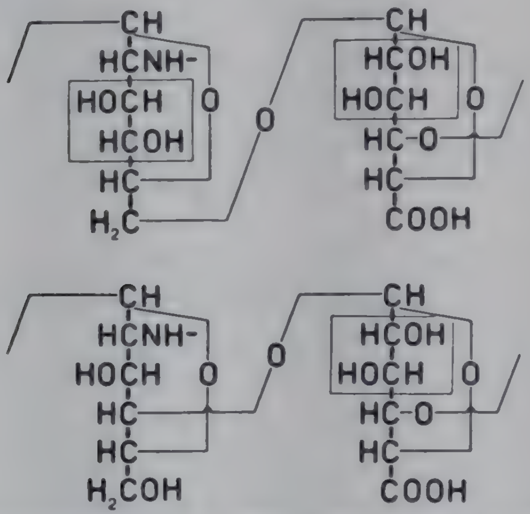


FIG. 1. A glucosaminoglucuronoside with two groups (above) and one group (below) reacting with periodic acid.

EXPERIMENTAL

Consumption of Periodic Acid by Some Mucopolysaccharides

The consumption of periodic acid was measured according to the method of Fleury and Fatôme (4).

50 mg. samples were dissolved in 5 ml. of water to which 3 ml. of a 0.11 N periodic acid solution were added. The samples were left standing for 20 hours at room temperature. After that time the excess of periodate was destroyed through addition of 10 ml. of a 0.049 N arsenous acid solution. Some potassium iodide was added and the arsenous acid not consumed was oxidized with 0.1 N iodine solution.

The consumption of periodic acid by some polysaccharides related to heparin, calculated on 100 mg. of ash-free organic substance, was as follows:

	S, per cent of dry substance	Periodic acid consumed, mg. HIO_4
Hyaluronic acid, Na salt		54.6
Heparin monosulfuric acid, Ba salt	5.42	52.8
Chondroitinsulfuric " Ca "	3.91	16.3
Heparin trisulfuric " Na "	12.17	8.2

The consumption of periodic acid by heparin monosulfuric acid equals that of the hyaluronic acid as calculated on organic substance. It is evident that heparin trisulfuric acid does not react with the periodic acid, the low figure found being partly due to the presence of pigments adhering to the heparin. Chondroitinsulfuric acid consumes a certain smaller amount of periodic acid but not more than can be explained through the splitting off of sulfate groups. The sulfur content of this preparation of chondroitinsulfuric acid was 3.91 per cent, whereas the calculated figure was 4.94 per cent. About 20 per cent of the sulfates had been split off during preparation, thus liberating α-glycol groups.

Color Test on Different Polysaccharides

The color reaction with fuchsin-sulfurous acid after periodate oxidation can, as well as the periodate consumption, be used as a qualitative test for glycol structures in sugars and for a rough quantitative estimation of polysaccharides. One of us (B. Å.) elaborated a technique for the quantitative colorimetric test in which the excess of periodic acid was destroyed through ultraviolet irradiation before the fuchsin-sulfurous acid was added.

The procedure was as follows: (1) Periodic acid, 400 mg. of H_5IO_6, and 135 mg. of $NaAc \cdot 3H_2O$ were dissolved in 50 ml. of water; pH about 2; (2) 1 N HCl; (3) fuchsin-sulfurous solution made up as follows: 2 gm. of basic fuchsin dissolved in 400 ml. of boiling water. To the cooled and filtered solution 10 ml. of 2 N hydrochloric acid were added and 4 gm. of potassium metabisulfite; this was decolorized with 1 gm. of charcoal. Up to 10 ml. or more of 2 N hydrochloric acid were added in small portions until, after the last addition, the mixture dried spontaneously in a thin film on a glass slide, without giving a pink color.

Method—The substance was dissolved in 5 ml. of water, and 3 ml. of the periodic acid solution were added, whereupon the sample was heated to boiling. The solution was immediately irradiated by a 500 watt quartz lamp in a 125 ml. quartz flask. The lamp, 20.5 cm. high, was placed 7 cm. above the flask and surrounded by an aluminum cylinder having a diameter of 18 cm. In order to prevent reduction of the periodic acid to iodine the lamp was not allowed to radiate with its full capacity. On irradiation with 1.8 amperes during 8 minutes, no free iodine was produced and the heat was very slight. After irradiation 2 ml. of 1 N HCl and 8 drops of the Schiff reagent were added. The color extinction was measured after 5 minutes in a Coleman spectrophotometer.

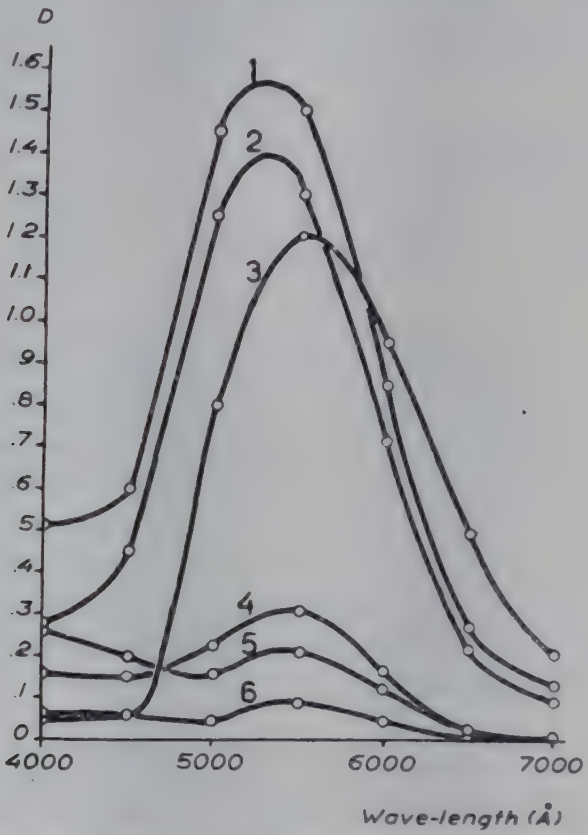


FIG. 2. Color intensity in the fuchsin-sulfurous acid reaction after periodate oxidation of some polysaccharides related to heparin. Air-dried samples. Curve 1, hyaluronic acid, sodium salt, 50 mg.; Curve 2, heparin monosulfuric acid, barium salt, 50 mg.; Curve 3, galacturonic acid, 50 mg.; Curve 4, chondroitinsulfuric acid, calcium salt, 100 mg.; Curve 5, heparin trisulfuric acid, sodium salt, 50 mg.; Curve 6, water.

The color intensity given in this reaction by the different mucopolysaccharides is shown in Fig. 2. As is seen, heparin trisulfuric acid and chondroitinsulfuric acid do not react with periodate, whereas heparin monosulfuric acid and hyaluronic acid are easily oxidized.

The color intensity obtained with hyaluronic acid is proportional to the amount of substance present (Fig. 3), a proportionality not to be found on analysis of heparin monosulfuric acid.

DISCUSSION

Oxidation with periodic acid has revealed definite differences between heparin monosulfuric acid and chondroitinsulfuric acid. There must be a difference either in the linkages between the single sugar components or in the location of the sulfate group. In accordance with this they also show a different behavior toward hydrolysis with acids. In the chondroitinsulfuric acid there cannot be more than one glycol unit with two adjacent hydroxyl groups. The sulfuric acid is probably bound to one of these hydroxyl groups, because as long as chondroitinsulfuric acid is intact, as it is

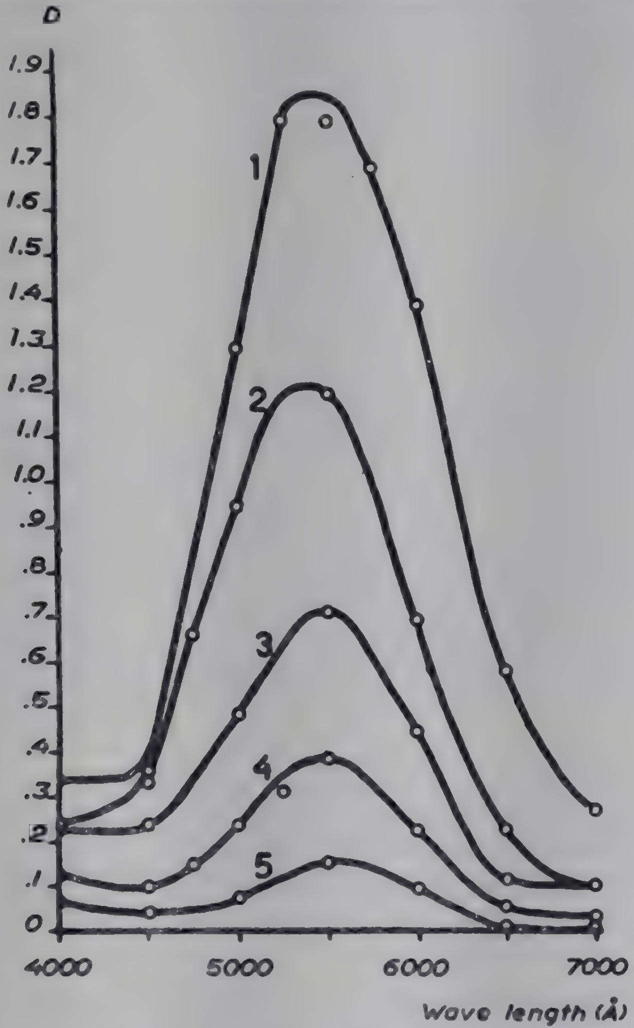


FIG. 3. The color intensity of different amounts of hyaluronic acid (sodium salt) in the fuchsin-sulfurous acid test after periodate oxidation. Calculated on ash-free organic substance. Curve 1, 30 mg.; Curve 2, 20 mg.; Curve 3, 10 mg.; Curve 4, 5 mg.; Curve 5, water.

in the cartilage, it does not react with periodic acid and gives a negative Schiff color reaction, whereas the ordinary preparations of chondroitinsulfuric acid consume a small amount of periodate. This is probably due to a loss of sulfate groups during preparation, as is also evidenced through the low sulfur content.

The Schiff reaction, if applied histologically, seems to enable us to demonstrate the presence of the heparin monosulfuric acid in tissues. Thus the granules of the tissue mast-cells in the subcutaneous tissue of the young rat give a very strong fuchsin-sulfurous acid reaction, which could be due to the presence of this acid or some other heparin precursor.

SUMMARY

Whereas ordinary heparin does not react with periodic acid, the heparin monosulfuric acid gives a color reaction with fuchsin-sulfurous acid after periodate oxidation.

The granules of the mast-cells in the subcutaneous tissue of the rat are strongly colored with Schiff's reagent, which could indicate the presence of heparin monosulfuric acid or some other heparin precursor. Chondroitinsulfuric acid is resistant to this reagent, the cartilage being completely unstained when treated with periodic acid and subsequently with fuchsin-sulfurous acid. This indicates a difference between chondroitinsulfuric acid and the heparin monosulfuric acid, either in the internal linkages between the two carbohydrate moieties or in the location of the sulfate groups.

BIBLIOGRAPHY

1. Charles, A. F., and Todd, A. R., *Biochem. J.*, **34**, 112 (1940).
2. Jorpes, J. E., and Gardell, S., *J. Biol. Chem.*, **176**, 267 (1948).
3. Hotchkiss, R. D., *Arch. Biochem.*, **16**, 131 (1948).
4. Fleury, P., and Fatôme, M. J., *Pharm. et chim.*, **21**, 247 (1935).

THE METABOLISM OF PLUTONIUM IN RATS FOLLOWING INTRAMUSCULAR INJECTION*

By KENNETH G. SCOTT, DOROTHY J. AXELROD, HARVEY FISHER,
J. F. CROWLEY, AND JOSEPH G. HAMILTON

(*From the Divisions of Radiology and Medicine, Crocker Radiation Laboratory,
and the Division of Medical Physics, University of California,
San Francisco and Berkeley*)

PLATES 1 AND 2

(Received for Publication, July 6, 1948)

The metabolism of plutonium in man has become a matter of concern with the development of the chain-reacting pile. For this reason a portion of the first plutonium produced by the Clinton pile was set aside for animal studies in order to evacuate the hazards which plutonium might present to man. In March, 1944, 11 mg. of plutonium were made available to this laboratory for animal studies from the first production of the Clinton pile. Later studies were undertaken at the Metallurgical Laboratory, University of Chicago (1), and at Los Alamos (2).

Plutonium is produced by the neutron irradiation of U^{238}; the U^{239} initially formed decays by the emission of a negative β-particle. The Ne^{239} created by this process in turn decays by negative β-particle emission to form Pu^{239}. This isotope of plutonium, having a half life of 24,000 years, decays by the release of α-particles to V^{235}, which occurs in nature and has a half life of 7×10^8 years.

Methods

Preparation of Material—The plutonium was prepared in the three valence states for injection by Professor R. Connick and his colleagues, working under the direction of Dean W. M. Latimer in the College of Chemistry.

The +3 valence state was prepared by the reduction of $PuCl_4$ by SO_2 in 0.47 N HCl. The +4 plutonium was prepared prior to the intramuscular injection by first precipitating the +4 hydroxide from the original $PuCl_4$ solution and dissolving the precipitate in 0.47 N HNO_3. The + 6 plutonium was prepared by oxidation of the $PuO_2(NO_3)_2$ with dichromate. The plutonium was precipitated as sodium plutonyl acetate, the preparation dissolved in HCl, and then precipitated as $NaPuO_2Ac_3$. The precipitate was again dissolved in HCl and the plutonium reprecipitated with NaOH. Finally, this precipitate was dissolved in 0.45 N HCl.

---

*This work was performed under contract No. W-7405-eng-48-A with the Manhattan District, United States Army.

The solutions containing the three separate valence states of plutonium were tested qualitatively by a spectrophotometric method and each was found to contain less than 20 per cent of plutonium in the form of valence states other than the one specifically desired. These stock solutions contained approximately 2 mg. of plutonium per cc. Immediately before injection the solutions were diluted with sufficient isotonic saline to bring the pH to 2.5, and they contained approximately 15 γ of plutonium per cc.

Administration to Rats—Owing to nutritional and metabolic similarities to man, the animal chosen for this work was the rat. The three plutonium solutions, as described above, were immediately administered to rats. In order to avoid undue plating of the plutonium on the syringes and needles used for injection, they were washed with 0.01 N HCl before use. In spite of this precaution, considerable difficulty was experienced with plating, especially with respect to Pu in the +4 state. However, the equipment used for injection was assayed for plutonium in order that the actual dose of Pu administered to the rats could be determined.

Twelve groups of three animals each were injected with approximately 15 γ of Pu intramuscularly in the left leg, the Pu solutions being employed in the three valence states described above. Following this, groups of rats were sacrificed at 4, 16, 64, and 256 days after administration of plutonium in the +3, +4, and +6 valence states.

Three groups of rats were given plutonium in its three valence states orally and sacrificed 4 days later.

The excreta were collected from all groups at daily intervals. In order to reduce the rather laborious plutonium assays as much as possible, the urine and feces were pooled for the following intervals, 0 to 2 days, 2 to 4 days, 4 to 8 days, 8 to 16 days, and every 8 days thereafter. When the animals were sacrificed, the organs removed included the liver, kidney, testes, spleen, muscle, skin, stomach, large and small intestine together with the cecum, bone, lung, brain, and blood. The left hind leg, which was the site of intramuscular administration, was removed and assayed separately in order to determine the percentage of plutonium remaining unabsorbed. Testes, spleen, muscle removed from the uninjected right leg, stomach, small and large intestines, lungs, brain, and 3 to 5 cc. of blood obtained by heart puncture were pooled for each group. In the case of bone, the uninjected right leg bones were assayed separately from the rest of the skeleton. The skinned carcass, which consisted of the rest of the skeleton, muscle, fat, and blood, was dried and ashed as a unit. The skeleton was separated from this by sifting the ash through a fine screen. The plutonium content of both of these portions was determined. The plutonium present in this ash, not included in the skeleton, is listed under "balance" in Tables I, III, and V after the calculated plutonium content of the muscle system of the animal had been subtracted from it.

The tissues were assayed in the following manner: Immediately after the animals were sacrificed, the organs and tissues were removed and weighed while wet. During the first part of the experimental program with plutonium, all of the tissues were ashed in an electric muffle following a preliminary drying at 150°. Several determinations were done in which a known amount of plutonium in its three valence states was added to inactive animal ash, which was brought up to a temperature of 500° and maintained at this level for 24 hours. No measurable amount of plutonium was lost from this ashing method by volatilization.

In later studies the tissues were wet ashed with concentrated HNO_3 and 30 per cent H_2O_2. When the ashing was completed, all of the acid was boiled off, leaving a white ash. Following either wet or dry ashing, the ash was dissolved in a solution which was 2 N HNO_3 and 0.2 M hydroxylamine. The final concentration of animal ash in the nitric acid hydroxylamine solution was 20 mg. per cc. 0.5 cc. of the solution was used for the assay of plutonium. Duplicate assays were made for every tissue and the details of the analytical procedure were as follows:

500 γ of $La(NO_3)_3$ in 25 microliters were added to 0.5 cc. of a solution of tissue ash in a 2 cc. centrifuge cone. (Because of the presence of α-emitting contaminants in some of the $La(NO_3)_3$ available, the latter must be checked for α emitters by running blanks on each batch received. The radioactive contamination present is believed to be actinium, whose chemical properties resemble closely those of lanthanum, as well as plutonium in the +4 state.)

0.2 cc. of 6 N HF was added and the mixture stirred with a platinum rod. The cone was then centrifuged at 6000 R.P.M. in a small centrifuge for 30 seconds. The cone was removed and the walls washed down with the supernatant solution. The mixture was centrifuged for 3 minutes and the supernatant solution discarded. The precipitate of LaF_3 containing the plutonium was then dissolved in concentrated nitric acid and transferred to a platinum dish 1 inch in diameter. The cone was washed with a small amount of water, and this was also added to the dish. 2 drops of 6 N HF were added to the solution in the platinum dish in order to reprecipitate the LaF_3 and the plutonium.

The dish containing the precipitate was gently warmed on a hot-plate until dry and then flamed to a dull red heat for a few seconds.

The precipitate was found to form a very thin even film which was quite adherent to the platinum. The α-particles from these samples were counted, each sample being counted twice. It was found that the LaF_3 precipitated from the diluted ash samples of bone, feces, and urine was somewhat more bulky than would be expected from the amount of lanthanum used as a carrier. A large series of α-particle measurements was made from the ash of these three types of material, and a relatively constant degree of self-absorption of the α-particles was found which ranged from 18 to 22 per cent for the feces and bone and 10 per cent for the urine. Owing to the fact that the remainder of this extraneous material in the LaF_3 precipitate would have necessitated a slow and laborious purification procedure, the appropriate correction factor was applied to these samples.

TABLE I

Deposition of +3 Plutonium in Tissues of Rat after Intramuscular Administration into Left Leg

The values given are in per cent of the dose. Average values for three rats at each time interval.

	4 days		16 days		64 days		256 days	
	per cent per organ	*per cent per gm.*	*per cent per organ*	*per cent per gm.*	*per cent per organ*	*per cent per gm.*	*per cent per organ*	*per cent per gm.*
Lungs	0.05	0.04	0.06	0.05	0.09	0.05	0.07	0.05
Spleen	0.08	0.13	0.09	0.15	0.22	0.40	0.21	0.42
Blood	0.45	0.04	0.20	0.02	0.10	0.01		
Liver	1.12	0.14	0.87	0.14	1.71	0.30	0.93	0.12
Kidney	0.30	0.18	0.10	0.07	0.27	0.19	0.31	0.17
Brain	<0.01*	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Stomach	0.06	0.02	0.03	0.02	<0.01	<0.01	0.09	0.03
Intestines	0.25	<0.01	0.23	0.03	0.08	<0.01	0.12	<0.01
Bone	10.8	0.64	18.7	1.13	30.3	2.09	30.6	1.97
Muscle	0.50	<0.01	0.76	<0.01	2.74	0.03	1.66	0.02
Balance	2.16		1.81		1.78		1.37	
Left leg	77.1		68.0		39.7		23.7	
Skin	0.61	0.03	0.62	0.03	0.49	0.02	0.20	<0.01
Gonads			0.06	0.02				
Tail					1.68	0.24		
Urine	0.11		0.19		0.97		1.12	
Feces	1.53		0.74		19.5		30.8	
Actual recovery	95.1		92.5		99.6		91.2	

* Although samples in this and the succeeding tables marked <0.01 per cent had measurable amounts of activity, the activities were of a low order and are probably not significant to more than two decimal places.

Bone radioautographs were prepared from undecalcified femur sections by techniques described elsewhere (3). These were made from animals sacrificed at intervals ranging from 8 to 256 days after intramuscular administration. Doses ranging from 15 to 25 γ were used.

Results

The average value of absorption of plutonium in each of its three valence states from the gastrointestinal tract was found to be 0.007 per cent. This value may be even lower, since it is predicated primarily upon the limits of the sensitivity of the counting apparatus used and the number of micrograms of plutonium available for these studies.

Data are presented in Tables I and II which give the relative deposition of plutonium in the tissues of the rat when administered in the +3 state. These studies were carried out for 256 days after administration of the plutonium, as were those involving the +4 and +6 valence states. It can be seen from an examination of Table I that +3 plutonium is only partly absorbed from the injection site, 77 per cent remaining at 4 days and 23 per cent at 256 days. The largest portion of the material absorbed is deposited in the skeleton. Liver, kidney, and spleen were the only other tissues examined which contained relatively large concentrations of plutonium. The excretion of plutonium occurred primarily in the feces. In Table II the same data are presented with corrections[1] for recovery and absorption from the injection site.

TABLE II

Deposition of +3 Plutonium in Tissues of Rat Corrected for Recovery and for Unabsorbed Balance at Injection Site

Average values for three rats at each time interval.

	4 days		16 days		64 days		256 days	
	per cent per organ	*per cent per gm.*	*per cent per organ*	*per cent per gm.*	*per cent per organ*	*per cent per gm.*	*per cent per organ*	*per cent per gm.*
Lungs	0.32	0.25	0.26	0.22	0.15	0.09	0.11	0.08
Spleen	0.50	0.82	0.40	0.66	0.38	0.69	0.32	0.64
Blood	2.84	0.25	0.88	0.09	0.17	0.02		
Liver	7.06	0.88	3.84	0.62	2.94	0.52	1.41	0.18
Kidney	1.89	1.13	0.44	0.31	0.46	0.33	0.47	0.26
Brain	0.03	0.02	0.03	0.02	0.02	<0.01	<0.01	<0.01
Stomach	0.38	0.23	0.13	0.09	0.01	0.01	0.14	0.05
Intestines	1.58	0.06	1.02	0.13	0.14	<0.01	0.18	<0.01
Bone	68.1	4.04	82.6	4.99	52.1	3.59	46.3	2.98
Muscle	3.15	0.04	3.36	0.04	4.71	0.05	2.51	0.03
Skin	3.85	0.19	2.74	0.13	0.84	0.03	0.30	0.01
Gonads			0.26	0.09				
Tail					2.89	0.41		
Urine	0.69		0.84		1.67		1.69	
Feces	9.65		3.27		33.5		46.6	

---

[1] These corrected values were obtained by calculating the relative amounts of plutonium present in all of the tissues and excreta, exclusive of the plutonium remaining unabsorbed in the left hind leg, and the amount of plutonium in the balance less the calculated quantity present in the blood, muscle, and carcass. This type of extrapolation, which attempts to correct for the amount of unabsorbed plutonium

Tables III and V demonstrate the deposition of plutonium in the tissues of the rat when administered as the +4 and +6 valence states. The corrected values are given in Tables IV and VI. These data are similar to those obtained for plutonium in the +3 valence state, demonstrating major deposition in the skeleton. Of all of the soft tissues, the liver, kidney, and spleen show the highest affinity for plutonium per gm. However, their concentration of plutonium per gm. averaged from one-fifth to one-tenth that of bone. The corresponding concentration of plutonium in the other soft tissues was very much less.

TABLE III

Deposition of +4 Plutonium in Tissues of Rat after Intramuscular Administration into Left Leg

The values given are in per cent of the dose. Average values for three rats at each time interval.

	4 days		16 days		64 days		256 days	
	per cent per organ	*per cent per gm.*	*per cent per organ*	*per cent per gm.*	*per cent per organ*	*per cent per gm.*	*per cent per organ*	*per cent per gm.*
Lungs	<0.01	<0.01	0.01	0.01	0.03	0.02	0.02	0.01
Spleen	<0.01	<0.01	0.01	0.03	0.03	0.07	0.05	0.12
Blood	0.05	<0.01	0.01	0.01	0.04	0.01		
Liver	0.08	<0.01	0.19	0.03	0.56	0.08	0.41	0.04
Kidney	0.03	0.02	0.08	0.04	0.10	0.05	0.07	0.03
Brain	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Stomach	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Intestines	0.05	<0.01	0.05	<0.01	0.11	<0.01	0.07	<0.01
Bone	1.44	0.08	2.18	0.14	12.9	0.84	11.5	0.60
Muscle	0.03	<0.01	0.08	<0.01	0.63	<0.01	0.59	<0.01
Balance	2.35		1.05		1.68		0.28	
Left leg	95.8		87.6		68.1		66.8	
Skin	<0.01	<0.01	0.06	<0.01	0.17	<0.01	0.21	<0.01
Gonads			<0.01	<0.01	0.05	0.02	0.06	0.02
Tail					0.84	0.13		
Urine	0.01		0.04		0.39		0.43	
Feces	0.11		0.43		4.70		9.42	
Actual recovery	100.0		91.8		90.3		89.9	

---

present, appears to be the best method of indicating the true metabolic behavior of this substance. The observed quantity of plutonium in the balance, less that estimated to be present in the blood and muscle of the carcass, is thought to represent the amount of the injected solution which extravasated beyond the point of amputation and remained unabsorbed.

For all groups it will be noted that the corrected data show that there is no apparent and significant difference in either the distribution or excretion of plutonium in its three valence states. This is demonstrated in Text-fig. 1 which shows the relative proportions of the absorbed plutonium in the three valence states in the excreta and in the skeleton. Their similarity suggests that plutonium exists in the body in the same valence state, regardless of the valence state in which it was administered.

TABLE IV

Deposition of +4 Plutonium in Tissues of Rat Corrected for Recovery and for Unabsorbed Balance at Injection Site

Average values for three rats at each time interval.

	4 days		16 days		64 days		256 days	
	per cent per organ	*per cent per gm.*	*per cent per organ*	*per cent per gm.*	*per cent per organ*	*per cent per gm.*	*per cent per organ*	*per cent per gm.*
Lungs	0.22	0.17	0.19	0.16	0.15	0.10	0.09	0.04
Spleen	0.33	0.50	0.32	0.95	0.15	0.34	0.22	0.53
Blood	2.76	0.22	0.19	0.01	0.19	0.01		
Liver	4.40	0.39	6.03	0.95	2.72	0.39	1.79	0.18
Kidney	1.66	1.10	2.54	1.27	0.49	0.24	0.31	0.13
Brain	0.04	0.03	0.02	0.02	<0.01	<0.01	0.04	0.03
Stomach	0.11	0.05	0.13	0.06	0.05	0.03	0.04	0.02
Intestines	2.76	0.28	1.59	0.16	0.15	0.04	0.31	0.02
Bone	79.3	4.40	69.3	4.44	62.7	4.09	50.3	2.63
Muscle	1.66	0.02	2.54	0.03	3.06	0.03	2.58	0.02
Skin	0.22	0.03	1.91	0.06	0.83	0.03	0.92	0.02
Gonads			0.32	0.10	0.24	0.10	0.26	0.09
Tail					4.09	0.63		
Urine	0.56		1.27		1.90		1.88	
Feces	6.06		13.6		22.9		41.2	

However, a very great difference in the amounts of plutonium absorbed from the site of injection was noted for the three valence states. The greatest degree of absorption was observed for +6 plutonium, the least for +4 plutonium, and +3 was intermediate in character. These dissimilar rates of uptake from the injection site are shown in Text-fig. 2.

Radioautographs

Radioautographs of adult rat femurs were made at periods ranging from 8 to 256 days after plutonium administration. All of these showed the deposition of plutonium on bone surfaces; *i.e.*, in the region of the periosteal and endosteal bone surfaces, and on the endosteal covering of the trabecular

TABLE V

Deposition of +6 Plutonium in Tissues of Rat after Intramuscular Administration into Left Leg

The values given are in per cent of the dose; average values for three rats at each time interval.

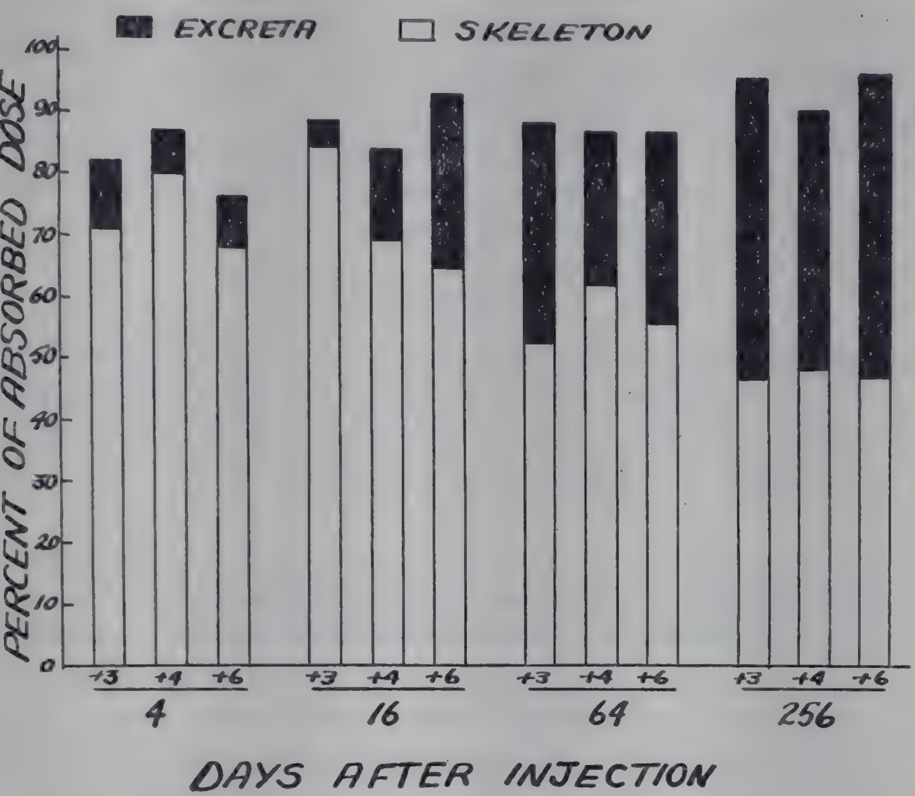
	4 days		16 days		64 days		256 days	
	per cent per organ	*per cent per gm.*	*per cent per organ*	*per cent per gm.*	*per cent per organ*	*per cent per gm.*	*per cent per organ*	*per cent per gm.*
Lungs	0.08	0.06	0.10	0.08	0.09	0.06	0.05	0.03
Spleen	0.10	0.16	0.22	0.40	0.28	0.47	0.20	0.34
Blood	0.86	0.07	0.21	0.02	0.11	<0.01		
Liver	3.58	0.47	1.89	0.34	2.24	0.30	1.09	0.13
Kidney	0.50	0.28	0.47	0.28	0.91	0.48	0.20	0.12
Brain	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Stomach	0.17	0.05	0.06	0.03	0.08	0.04	0.03	0.01
Intestines	0.59	0.03	0.50	0.05	0.26	0.02	0.09	<0.01
Bone	17.2	0.83	43.7	1.78	28.7	1.96	34.6	2.31
Muscle	0.65	0.01	1.56	0.02	0.83	0.01	0.73	0.01
Balance	4.73		1.50		3.57		2.71	
Left leg	70.4		30.0		33.6		12.6	
Skin	0.56	0.02	0.60	0.02	0.60	0.02	0.26	0.01
Gonads			0.10	0.04				
Tail					1.72	0.28		
Urine	0.10		5.51		2.30		3.28	
Feces	1.95		13.4		14.1		32.4	
Actual recovery	101.5		99.8		90.4		88.3	

TABLE VI

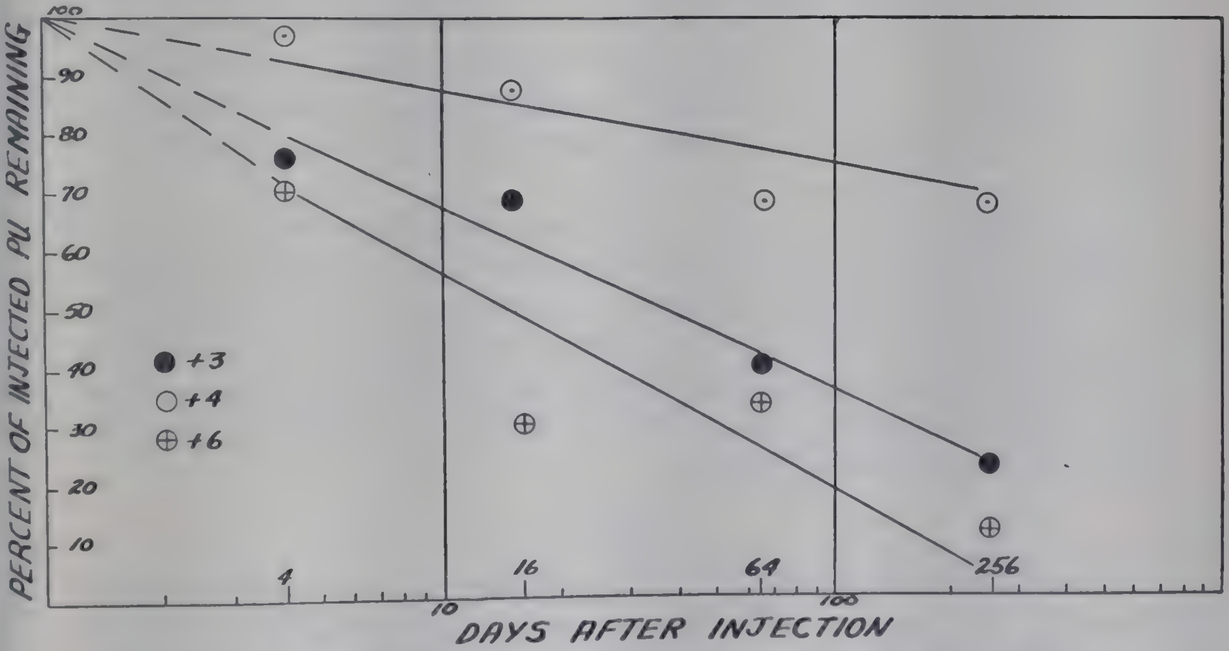
Deposition of +6 Plutonium in Tissues of Rat Corrected for Recovery and for Unabsorbed Balance at Injection Site

Average values for three rats at each time interval.

	4 days		16 days		64 days		256 days	
	per cent per organ	*per cent per gm.*	*per cent per organ*	*per cent per gm.*	*per cent per organ*	*per cent per gm.*	*per cent per organ*	*per cent per gm.*
Lungs	0.30	0.23	0.15	0.12	0.17	0.11	0.07	0.04
Spleen	0.38	0.61	0.32	0.59	0.53	0.88	0.27	0.47
Blood	3.27	0.27	0.31	0.03	0.21	0.02		
Liver	13.6	1.78	2.77	0.50	4.21	0.56	1.49	0.18
Kidney	1.90	1.06	0.69	0.41	1.71	0.90	0.27	0.16
Brain	0.03	0.02	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Stomach	0.65	0.19	0.09	0.04	0.15	0.08	0.04	0.01
Intestines	2.24	0.11	0.73	0.07	0.49	0.04	0.12	<0.01
Bone	65.3	3.15	64.0	2.61	55.8	3.68	47.4	3.17
Muscle	2.47	0.03	2.28	0.03	1.56	0.02	1.00	0.01
Skin	2.13	0.08	0.88	0.03	1.13	0.04	0.36	0.01
Gonads			0.15	0.06				
Tail					3.23	0.53		
Urine	0.38		8.07		4.32		4.50	
Feces	7.40		19.6		26.5		44.4	



TEXT-FIG. 1. Proportion of plutonium found in excreta and skeleton of the rat after intramuscular administration of plutonium in +3, +4, and +6 valence states. Values corrected for absorption from the left leg.



TEXT-FIG. 2. Loss of plutonium from the hind left leg of the rat after intramuscular injection of the +3, +4, and +6 valence states. The ordinate scale gives the per cent of plutonium remaining at the injection site.

bone (Fig. 1). The picture of plutonium deposition at 8 days was the same as that found after 256 days. In other words, no shifting or redistribution of plutonium took place after its initial deposition in the adult animal. Fig. 2 shows a section in which the periosteum was fortuitously stripped off of the bone. The radioautograph demonstrates that a large amount of plutonium is present in this stripped layer.

Conclusions

No significant variations in the metabolic behavior of plutonium were observed in each of its three valence states. It is probable that plutonium, when absorbed and distributed throughout the body, exists in a single valence state which is independent of its valence at the time of administration.

Plutonium is not absorbed from the gastrointestinal tract to any significant degree. Following parenteral administration, the chief organ of deposition is the skeleton. The soft tissues having the greatest concentration of plutonium are liver, kidney, and spleen; their content of plutonium per gm., however, is from one-fifth to one-tenth that of bone.

The chief channel of elimination is the digestive tract. The rate of plutonium excretion is very slow and its half period of retention in the body is estimated to be greater than 2 years.

Radioautographic studies show that plutonium is deposited primarily in the region of the periosteum, endosteum, and the endosteal covering of the trabecular bone. Examination of many of these preparations suggests that the principal site of deposition is on the bone surface and that a very limited accumulation takes place in the mineral structure of the bone. No significant redistribution of plutonium took place in the bone during the period of these experiments.

The selective localization of the plutonium on the bone surfaces explains the high toxicity of plutonium compared to equivalent quantities of radium. This effect arises from the fact that the radium is distributed throughout the mineral portion of the bone, and a large proportion of the α-particles is absorbed before they can enter the marrow cavity. Plutonium α-particles can bombard bone marrow more readily, since there is relatively less self-absorption of radiation by the bone.

SUMMARY

Detailed metabolic studies of Pu^{239} administered to rats intramuscularly in the +3, +4, and +6 valence state are presented. Plutonium was not found to be absorbed from the gastrointestinal tract to any extent. The skeleton was the main organ of deposition of plutonium, and the degree of retention in this organ was very great. No significant differences were

observed in the metabolic properties of plutonium absorbed by the body for its three valence states. Radioautographs demonstrate the deposition of plutonium in the region of the endosteum, periosteum, and the endosteal covering of the trabecular bone.

BIBLIOGRAPHY

1. Finkle, R. D., *et al.*, MDDC-1140, Technical information division, Oak Ridge directed operations, U. S. Atomic Energy Commission.
2. Carritt, J., Fryxell, R., Kleinschmidt, J., Kleinschmidt, R., Langham, W., San Pietro, A., Schaffer, R., and Schnap, B., *J. Biol. Chem.*, **171**, 273 (1947).
3. Axelrod, D. J., *Anat. Rec.*, **98**, 19 (1947).

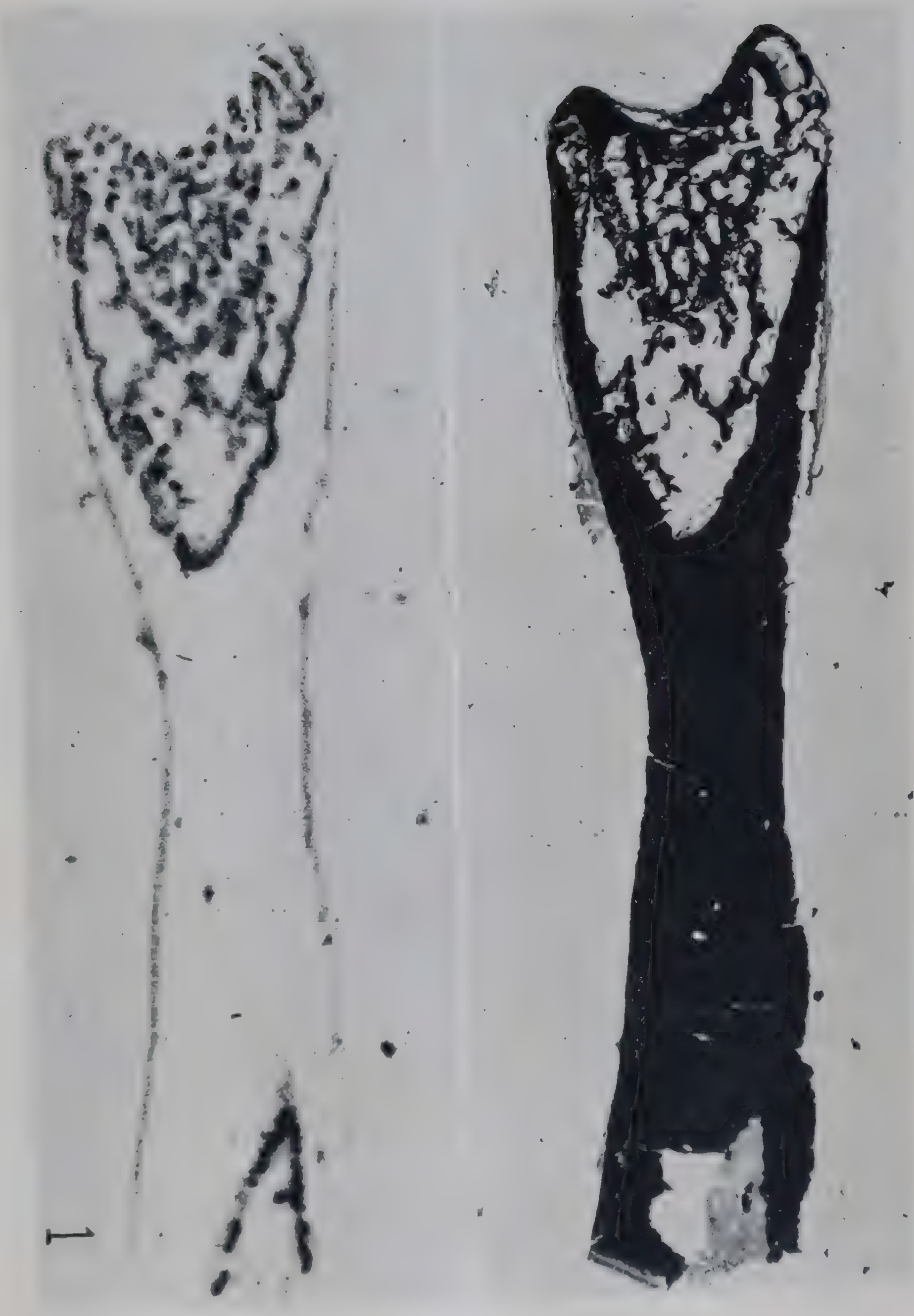
EXPLANATION OF PLATES

Plate 1

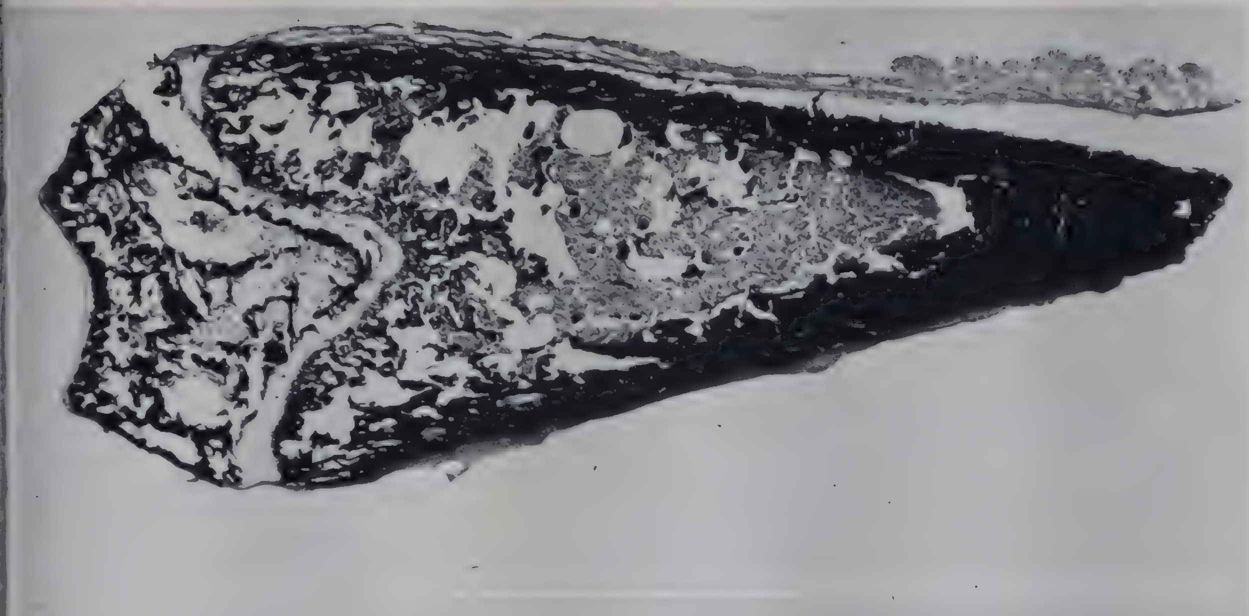
Fig. 1. Section of femur from an adult rat injected intramuscularly with 15 γ of plutonium and sacrificed after 8 weeks. The radioautograph demonstrates the superficial deposition of plutonium in the region of the periosteum, endosteum, and trabecular bone. Hematoxylin, eosin, and silver nitrate; × 8.

Plate 2

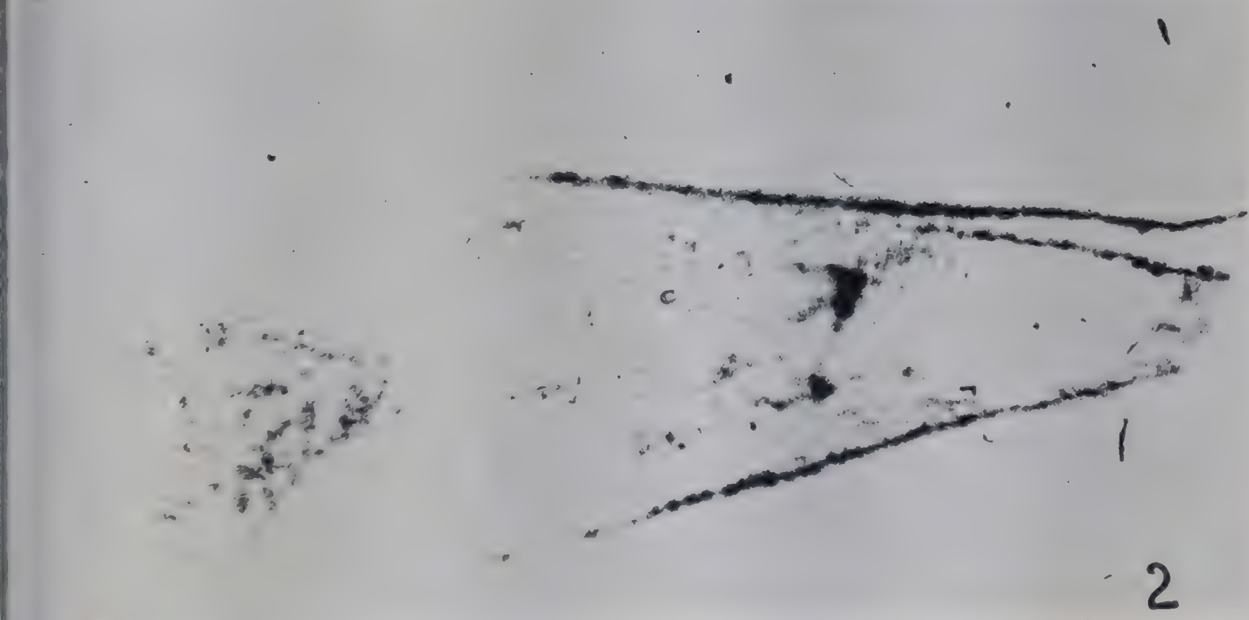
Fig. 2. Radioautograph and section of femur from a rat injected intramuscularly with 25 γ of plutonium and sacrified after 5 weeks. During the preparation of the sections the periosteum became separated from the shaft, and the autograph shows a heavy deposit of plutonium in this stripped layer. No calcium was detected in this particular area. Hematoxylin, eosin, and silver nitrate; × 10.4.



(Scott, Axelrod, Fisher, Crowley, and Hamilton: Plutonium)



1



2

(Scott, Axelrod, Fisher, Crowley, and Hamilton: Plutonium)

A STUDY OF THE MECHANISM OF "PHOSPHOTRANSFERASE" ACTIVITY BY USE OF RADIOACTIVE PHOSPHORUS*

By BERNARD AXELROD

(From the Enzyme Research Division, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture, Albany, California)

(Received for publication, June 21, 1948)

"Phosphotransferase" activity, which has been previously described (1), involves the enzymic transfer of phosphate from various aryl phosphates to particular hydroxyl compounds, apparently without the mediation of a nucleotide. It was indicated in this work that the phosphate group was transferred directly from the donor to the acceptor compound. The main basis for this belief was the observation that the enzyme plus inorganic phosphate did not transfer phosphate to the acceptor compound. Nevertheless it remained possible that the accepted phosphate arose from the pool of inorganic phosphate (which is always present since "phosphotransferase" activity is always accompanied by phosphatase activity), and the cleavage of the donor phosphate compound only provided the energy for the esterification.

It has now been possible through the use of P^{32} to prove the direct transfer of phosphate from donor to acceptor. This has been shown in two ways. When unlabeled *p*-nitrophenyl phosphate was used as a phosphate donor in the presence of labeled inorganic phosphate, the synthesized phosphate ester contained virtually no labeled phosphate. But when the same donor contained labeled phosphate and was used with unlabeled inorganic phosphate, the synthesized ester did contain labeled phosphate as active as that present in the donor.

In summary, the tests were conducted by subjecting *p*-nitrophenyl phosphate to the action of purified citrus phosphatase (which exhibits "phosphotransferase" activity very markedly) in the presence of methyl alcohol and inorganic phosphate. After incubation the increase in free nitrophenol was measured, showing the amount of nitrophenyl phosphate split. At the same time the increase in inorganic phosphate was measured to determine how much of the phosphate split from the substrate had been reesterified to methyl phosphate, as explained in the earlier paper (1). The methyl phosphate thus formed was isolated from the reaction mixture, and the specific radioactivity of the phosphorus was determined. As this value is independent of the amount recovered, considerable loss in yields was taken in order to insure high purity of the isolated product. Results are compared in terms of specific activity (counts per minute per mg. of P); nevertheless the yields of purified methyl phosphate were large enough to account for about a quarter of the synthesis calculated from the analyses of the digest.

---

* Enzyme Research Laboratory Contribution No. 112.

Preparation of Labeled $POCl_3$—The labeled $POCl_3$ was made by a method somewhat similar to that reported by Lindberg (2). A mixture of 6.29 gm. of 85 per cent H_3PO_4 and 1 ml. of a dilute aqueous solution of $Na_2HP^{32}O_4$ was aerated on a hot-plate until the decrease in weight showed that the solution was anhydrous, then thoroughly mixed with 15 gm. of PCl_5, and heated to give a yield of 80 per cent (based on PCl_5) of redistilled $POCl_3$.[1] Starting with 1.1×10^6 counts per minute, 3.13×10^5 counts per minute were recovered; the maximum possible recovery was 3.76×10^5.

Synthesis of Labeled p-Nitrophenyl Phosphate—The procedure previously used for the synthesis of inactive *p*-nitrophenyl phosphate was employed with some modification. To 310 mg. of *p*-nitrophenol, dissolved in 1.4 ml. of dry $CHCl_3$, 1.0 ml. of labeled $POCl_3$ was added, followed by 0.34 ml. of dry pyridine, care being taken to provide good agitation and cooling. After 30 minutes several pieces of ice were added and the reaction mixture allowed to stand several hours to insure decomposition of the acid chlorides. The *p*-nitrophenylphosphoric acid was obtained as a sticky tar by extraction with $CHCl_3$ and removal of the solvent with a stream of air. The crystalline sodium salt of the acid was obtained by adding 2 ml. of H_2O and enough sodium alcoholate to make the solution just alkaline to phenolphthalein, followed by the addition of a large excess of 1:1 acetone and ethanol. The crystals, after washing with 1:1 ethanol-ether, weighed 100 mg. As a further step in purification, the product was converted to the barium salt by dissolving it in 3 ml. of H_2O, adjusting the pH to the pink of phenolphthalein, and adding a slight excess of barium acetate. A slight precipitate was removed by centrifugation, and the supernatant was made 78 per cent with respect to ethanol. The resulting precipitate was washed twice with the same volume of 95 per cent ethanol and finally with ethyl ether. P content, found 7.56 per cent; calculated (for the trihydrate) 7.60 per cent. These crystals were found to be crystallographically identical with barium *p*-nitrophenyl phosphate prepared from an authentic sample of disodium *p*-nitrophenyl phosphate$\cdot 2H_2O$. Approximately 100 mg. of the crystals were diluted with the barium salt derived from 500 mg. of inactive disodium nitrophenyl phosphate for use in the following experiment.

---

[1] For the most efficient utilization of radioactive material one should use the H_3PO_4 as the limiting starting material, in accord with the stoichiometric proportions of 1 mole of H_3PO_4 to 3 moles of PCl_5 as determined by Geuther (3).

"Phosphotransferase" Reaction with Labeled Nitrophenyl Phosphate—454 mg. of the barium salt were converted quantitatively to the sodium salt and mixed with an equivalent quantity of ordinary sodium phosphate. Acetic acid was added to pH 5.0, also 9 cc. of methanol, 6 cc. of M acetate buffer (pH 5.0), and finally enzyme, to a total volume of 62 cc. The enzyme employed was 708 units of navel orange juice phosphatase, Preparation D (4). After 6 hours at 38°, tests showed that 91.2 per cent of the original nitrophenyl phosphate had been digested, with the appearance of only 28.6 per cent of the cleaved phosphate in the inorganic form, thus indicating that 22.4 mg. (71.4 per cent) of the original phosphorus had been reesterified to methyl phosphate.

Methyl phosphate was thereafter isolated from the reaction mixture as $BaMePO_4 \cdot H_2O$, essentially as described in the earlier paper (1). Two consecutive crystallizations from hot water were employed. 80.3 mg. of barium methyl phosphate (dried *in vacuo* at 56° to constant weight) were recovered.

The specific radioactivity of the phosphorus was found to be 29.2 ± 0.5 counts per minute per mg. of P in the initial barium nitrophenyl phosphate and 29.1 ± 0.5 in the recovered barium methyl phosphate.

"Phosphotransferase" Reaction with Radioactive Inorganic Phosphate—An even better proof of the direct transfer of phosphate from the donor to methyl alcohol is shown by the fact that in the presence of relatively large amounts of labeled inorganic phosphate the synthesized methyl phosphate was almost free from labeled phosphate. The reaction mixture contained 100 mg. of Na_2-*p*-nitrophenyl phosphate dihydrate, 3 ml. of methanol, 2 ml. of M acetate buffer, pH 5.0, 168 units of *p*-nitrophenyl phosphate phosphatase (lyophilized navel orange juice phosphatase Preparation D), 0.1 ml. of labeled phosphate containing 0.678 mg. of P and having 5.84×10^5 counts, and water to give a final volume of 21 ml. The pH was adjusted to 5.0 with acetic acid. Digestion was carried out at 38° for 5 hours, following which the solution was treated in essentially the same manner as before for the isolation of the methyl phosphate. In this case, however, after the reaction was stopped, inactive Na_2MePO_4, equivalent to 500 mg. of $BaMePO_4 \cdot H_2O$, was added to facilitate the recovery of the enzymically synthesized methyl phosphate, and 910 mg. of Na_2HPO_4 were added to dilute the labeled inorganic phosphate and minimize contamination of the methyl phosphate. After two crystallizations the specific activity did not change with further recrystallization. The final product weighed 121.4 mg. after drying over P_2O_5 at 56° *in vacuo* and had a count of 368 per minute. Analyses showed that 38.6 mg. of nitrophenol had been liberated during the reaction but only 3.71 mg. of inorganic phosphorus (equivalent to 43.3 per cent of the total nitrophenyl phosphate cleaved). The esterified

phosphorus corresponded to 41.8 mg. of $BaMePO_4 \cdot H_2O$. Since 500 mg. of that substance had been added as carrier, complete recovery of $BaMePO_4$ should have yielded 541.8 mg. The total count of the $BaMePO_4 \cdot H_2O$ (for the 41.8 mg. of synthesized phosphate ester) was therefore 1640 or a specific activity of 336 counts per minute per mg. of P. Had the phosphorus of the synthesized compound come from (or through) the inorganic pool, its specific activity could not have been less than 6.4×10^4, even in the least favorable (and really impossible) case in which all the phosphate was split from nitrophenyl phosphate before any of the methyl ester was synthesized.

In another experiment carried out under similar conditions the relative specific activity found for the synthesized $BaMePO_4 \cdot H_2O$ was 125 as compared to the hypothetical lower limit of 1.3×10^5. The contribution from the inorganic pool is therefore regarded as negligible.

SUMMARY

By using, in one case, P^{32}-labeled nitrophenyl phosphate, and, in another, radioactive inorganic phosphate it has been shown that in the "phosphotransferase"-catalyzed transfer of phosphate from nitrophenyl phosphate to methanol the transported phosphate does not pass through the inorganic stage.

It is a great pleasure to the author to acknowledge the kindness of Professor D. M. Greenberg and Dr. Theodore Winnick for their helpful advice and for making available the facilities of the Department of Biochemistry, University of California, for a portion of this work and to express thanks to Dr. F. T. Jones of the Western Regional Research Laboratory for the crystallographic analysis.

BIBLIOGRAPHY

1. Axelrod, B., *J. Biol. Chem.*, **172**, 1 (1948).
2. Lindberg, O., *Ark. Kemi, Mineral. o. Geol.*, **23 A**, No. 2 (1946).
3. Geuther, A., *J. prakt. Chem.*, **8**, 359 (1874).
4. Axelrod, B., *J. Biol. Chem.*, **167**, 57 (1947).

STUDIES ON THE PHOSPHORUS METABOLISM OF GREEN ALGAE AND PURPLE BACTERIA IN RELATION TO PHOTOSYNTHESIS

By HOWARD GEST* AND MARTIN D. KAMEN

(From the Mallinckrodt Institute of Radiology and the Department of Chemistry, Washington University, St. Louis)

(Received for publication, April 28, 1948)

The concept of circulating phosphate bond energy has been proposed as a basic mechanism for energy transfer in the chemosynthetic and photosynthetic reduction of CO_2 (1–4). Participation of phosphorylated intermediates in the "light" reactions of intact photosynthetic microorganisms has not been established, although several attempts have been made to demonstrate such phenomena (2, 5). To date, experiments with living cells show that a significant reproducible net change in internal phosphorus distribution is generally difficult to establish, even in cases in which the metabolism is known to involve phosphorylated intermediates. Hence, it is necessary either to prepare extracts capable of performing partial reactions in which intermediates accumulate or to study phosphorus turnover with a radioactive tracer (P^{32}).

The preparation of extracts having the capacity of reducing CO_2 under the influence of light has not yet been successful. We have therefore investigated the phosphorus turnover. The results obtained in this investigation indicate either that phosphorylation is an integral feature of the photosynthetic process proper or that non-related "dark" phosphorylative reactions are greatly stimulated in some way as a consequence of illumination. The existence of a true light-stimulated respiration or fermentation accompanied by gas consumption or production is considered to be very unlikely (6). However, the possibility that other "secondary" phosphorylative reactions not associated with CO_2 reduction are indirectly affected by illumination has not been eliminated conclusively.

In the course of the experiments, it has been found that the total phosphate content and its distribution in the cells are strongly dependent on the inorganic phosphate content of the growth medium. Cells grown in "high" phosphate media store an appreciable quantity of soluble phosphate which is readily lost when they are suspended in water, saline, and other solutions. The removal of a large fraction of this excess phosphate by washing has no apparent effect on the ability to carry on photosynthesis. These incidental observations on phosphate nutrition are of importance to the problems of photosynthesis only in so far as their cognizance is desirable for intelligent planning of future experiments. They are perhaps of more interest as an illustration of a phenomenon which appears to be rather wide-spread among many types of microorganism.

---

* Predoctoral Fellow of the American Cancer Society, 1947–48.

EXPERIMENTAL

Preparation of Cultures—Two species of Chlorococcales were used, *Chlorella pyrenoidosa* and *Scenedesmus* D_3 (Gaffron).[1] Both organisms were grown in pure culture in the medium described below (a modification of Gaffron's medium (7)): Part A, tap water 1000 ml., NH_4NO_3 0.52 gm., $MgSO_4 \cdot 7H_2O$ 0.52 gm., and KH_2PO_4 0.208 gm. (0.0125 gm. for the "low" P medium); Part B, tap water 100 ml., $KHCO_3$ 5 gm., and $NaHCO_3$ 5 gm.

Parts A and B were sterilized separately by autoclaving (Part A in bubbling flasks of the Warburg type). 10 ml. of Part B were added to each 240 ml. of Part A under sterile conditions to furnish the complete growth medium. The inoculated flasks were maintained in a water bath at 20–25°, aerated continuously with 5 per cent CO_2-95 per cent air-gas mixture, and illuminated with incandescent lamps. Cultures were ordinarily harvested for use 3 to 5 days after inoculation (not longer than 1 day after the density appeared to be maximal). Several experiments were made with cells grown in a medium as above with the addition of $\sim 10^{-5}$ M $FeCl_3$ and trace elements as recommended by Arnon (8). Cells grown in this supplemented medium showed no significant differences in phosphate turnover compared with cultures grown in the usual manner.

Rhodospirillum rubrum (strain SI) was grown anaerobically in glass-stoppered bottles with a yeast autolysate-mineral salt medium similar to that described by van Niel (9), with sodium acetate or *n*-butanol as hydrogen donor. Cultures were grown in media containing different concentrations of phosphate; these are indicated in connection with the results of the particular experiments. The bottle cultures were incubated at ~25° under constant illumination with incandescent lamps.

Manometric Measurements—Rates of photosynthesis and respiration of the algae were determined by measuring the rate of oxygen exchange in 0.1 M $NaHCO_3$ (*Scenedesmus*) or Warburg's Buffer 9 (*Chlorella*) with air as the gas phase. Respiratory measurements were made either by covering the bath with a dark cloth or by wrapping the individual vessels with aluminum foil. The foil is very effective in excluding light and was particularly useful in the P^{32} turnover experiments, in which it was desirable to run dark and light samples simultaneously in the same bath. Saturation light intensity was provided by a bank of 60 watt lamps above the vessels. The temperature was maintained at 25° or 30°, as indicated. It should be noted that rather dense suspensions were used in order to facilitate the P^{31} analyses. There must have been considerable "shading" in the light, since dilution of the suspensions usually increased the ratio, O_2 produced in the light-O_2 consumed in the dark.

---

[1] We are indebted to Dr. C. B. van Niel of Stanford University for supplying the strains of algae and purple bacteria used for these experiments.

The photosynthetic CO_2 assimilation by *Rhodospirillum rubrum* was followed with bacteria suspended in 0.05 per cent $NaHCO_3$ under an atmosphere of 5 per cent CO_2 in N_2 (10, 11). Sodium butyrate or *n*-butanol served as hydrogen donor. Changes in the bicarbonate content of the liquid phase were determined in control vessels by tipping in H_2SO_4 at the beginning and end of the experiment. Gas production during the dark "autofermentation" was measured in a similar manner in foil-wrapped vessels.

Phosphorus Determinations—Total phosphate was determined as inorganic orthophosphate after digestion of the material with H_2SO_4 and H_2O_2. After clearing the sulfuric digests with several drops of 30 per cent H_2O_2, they were diluted with distilled water and boiled vigorously to hydrolyze pyrophosphate. Aside from the use of Pictol (Mallinckrodt; in $Na_2SO_3 + NaHSO_3$ solution) in place of the usual reducing agents, the method of Lohmann and Jendrassik was followed (12). Optical densities were measured in a Klett-Summerson photoelectric colorimeter (Filter 66) after approximately 1 hour's development. Inorganic phosphate standards were always included. Differences in P^{31} assay of the order of 5 to 10 per cent were not considered significant, even when there appeared to be a "trend" in the data.

Inorganic phosphate in trichloroacetic acid (TCA) extracts was estimated by the Fiske-Subbarow method (13) with Pictol as the reducing agent.

Tests for labile phosphate esters were made by the procedure recently developed by Lowry and Lopez (14). With ascorbic acid as the reducing agent in acetate buffer solution at pH 4, presumably only true inorganic phosphate is detected by this procedure. Differences between the values obtained by this method and those shown by the Fiske-Subbarow method are ascribed to labile esters (acyl phosphate, etc.).

Radioactivity Measurements—Aliquots of the sulfuric acid digests were also used for assay of P^{32}. All samples (saline washes, etc.) were neutralized to the phenolphthalein end-point and evaporated to dryness on small watch-glasses under infra-red lamps. Activity was determined by means of a conventional end-window Geiger-Müller counting tube connected with a scale-of-64 circuit. Corrections for decay or self-absorption were unnecessary. In all experiments radioactivity balances were obtained and recoveries of labeled phosphate summed up over all chemical fractions as compared with the initial labeled content of the whole cells were always greater than 90 per cent.

Fractionation of Cells—Thus far only crude fractionations have been made, primarily because of difficulties which are discussed below. In the "uptake" experiments, bacteria or algae were exposed to solutions containing P^{32} (2 to 5 microcuries per ml.) in the form of inorganic phosphate under the same conditions as for manometric measurements. Each vessel usually contained ~0.1 ml. (wet volume) of cells. In a number of experiments with the algae small Erlenmeyer flasks (25 ml.) were used rather than Warburg vessels. In these instances, control manometric measurements were simultaneously made with aliquots of the algal suspension in order to be sure that the cells were photosynthesizing and respiring normally.

After suitable intervals of time, the suspensions were extracted directly or the cells washed and then extracted with ~10 ml. of cold TCA (final concentration 5 to 6 per cent) for about 1 hour at 5°. For the usual uptake experiment, the organisms were harvested and used without washing; the TCA extract was discarded, and the residue washed twice with 10 ml. portions of cold 5 per cent TCA. Specific activity (counts per minute per microgram of P^{31}) in the gross residue was then determined.

Fractionation of the TCA-insoluble residue was accomplished by a procedure developed for analysis of the same fraction of yeast.[2] As applied here, this consisted of the following steps. Lipides were removed with cold alcohol and subsequent repeated extractions with hot ether-alcohol mixture (3:1). The residue from the lipide extraction was then treated with 1 N KOH for 24 hours at 37°. This treatment brings phosphoproteins and nucleic acid phosphate into solution. Specific activities in the lipide fraction, KOH extract, and KOH residue were determined in the usual manner.

When the cells could be washed after exposure to P^{32}, the TCA extracts were retained for specific activity determinations (in these instances, the residue was washed only once with several ml. of 5 per cent TCA and the wash added to the extract).

Results

Phosphorus Exchange between Cells and Medium—Resting cell suspensions are ordinarily prepared by harvesting a culture and washing the cells thoroughly in order to remove traces of nutrients and other substances remaining from the occluded culture fluid. The washing liquid is usually distilled water, 0.85 per cent saline, or less frequently fresh culture medium. It is well known that substances must be readily lost from bacterial cells, since washing frequently leads to "inactivation" of the organisms (15). In spite of this possibility, washing is almost invariably necessary in P^{32} uptake experiments, since an excess of the tracer generally remains in the external medium. Consequently, the effect of washing on the internal phosphate composition was studied. Table I illustrates typical data obtained when organisms grown in the presence of P^{32} are washed with saline and phosphate-containing solutions. The phosphate in all fractions of such cells is necessarily labeled and completely equilibrated with exogenous phosphate; *i.e.*, external and internal phosphate have the same specific activity.

---

[2] Juni, E., Kamen, M. D., Reiner, J. M., and Spiegelman, S., in press.

TABLE I

Leakage of P^{32} from Internally Labeled Cells of Chlorella pyrenoidosa and Rhodospirillum rubrum (SI) into Successive Washes of Saline and Growth Medium at 0°

The results are expressed in counts per minute.

Experiment A, 5 day culture; Experiment B, 4 day culture; Experiment C, 5 day culture; Experiment D, 8 day culture. The washes consisted of 25 ml. portions of ice-cold solution. Centrifugations were made in a refrigerated centrifuge at 0° as rapidly as possible. In all cases, the saline was 0.85 per cent. For *Chlorella*, the medium was Part A of the growth medium (approximately 50 γ of P per ml). For *Rhodospirillum*, the medium was the same as the complete growth medium (700 to 800 γ of P per ml.). In Experiment B, approximately 0.3 ml. wet volume of algae was washed in each case. In Experiment C, 87 mg., dry weight, of bacteria were washed in each series.

	Chlorella pyrenoidosa				*Rhodospirillum rubrum* (SI)		
	Experiment A		Experiment B		Experiment C		Experiment D
	Saline	Medium	Saline	Medium	Saline	Medium	Medium
Wash 1	625,000	326,000	1,040,000	800,000	382,000	117,000	330,000
" 2	401,000	179,000	343,000	425,000	117,000	8,350	40,600
" 3	162,500	140,000	35,400	77,500	36,000	5,130	17,100
" 4	58,500	88,000	21,000	32,000	12,800	5,650	15,000
" 5	22,800	80,500	16,100	16,000	1,800	5,450	10,200
Washed organisms	2,420,000	2,850,000	1,847,500	1,806,000	374,000	683,000	1,125,000
Original supernatant per ml.	238,800	238,800	410,000	410,000	232,000	232,000	166,000

From these data, it is evident that internal phosphate is gradually removed by successive washes under conditions in which metabolism is presumably at a low level (0°). Similar results were found in experiments with *Scenedesmus* D_3.

The effects of washing on P^{31} distribution in *Rhodospirillum rubrum* are shown in Table II. The data were obtained by suspending the washed bacteria from Experiment C of Table I in 15 ml. of saline and extracting 1 ml. aliquots with TCA. Equal quantities of cells were used in each series (87 mg., dry weight).

If the specific activity in the external medium and the total number of counts associated with the cells originally (washes plus washed cells) are known, it is possible to make a fairly accurate estimate of the amount of P^{31} initially present in the bacteria (about 177 γ per ml.). Further, if we assume that the acid-insoluble phosphate does not exchange with the medium, as appears to be the case from the specific activities given in Table II, then it is clear that saline washes removed $\sim$87 per cent of the phosphate initially in the soluble fraction, whereas washing with medium increased the phosphate content of this portion by 49 per cent. The increase in phosphate content in the latter case is a *net* increase, since a considerable amount of the original phosphate was lost as shown by appearance of P^{32} in the washes. Similar experiments with *Scenedesmus* D_3 and *Chlorella pyrenoidosa* showed the same phenomenon; *i.e.*, cells grown in P^{32} can be differentially labeled simply by washing with unlabeled phosphate-containing solutions (room temperature).

TABLE II

Distribution of P^{31} and P^{32} in Rhodospirillum rubrum (SI) after Washing with Saline and Medium

	Saline-washed		Medium-washed	
	γ	*counts per min. per γ*	γ	*counts per min. per γ*
Total P*	75.7	329.0	321.5†	141.7
P in TCA extract	15.3	326.0	254.5	108.5
" " " residue	60.4	335.0	51.8	345.0

Specific activity in original supernatant $\cong$ 356 counts per minute per microgram (651 γ of P^{31} per ml.).

* Direct determinations, per ml. of suspension.

† Of the 321.5 γ, 81 γ of specific activity 6.5 were *not* associated with the cells.

Algae which have been washed with saline still show apparently normal photosynthetic and respiratory activity in spite of considerable loss of phosphate. This suggested that the "excess" soluble phosphate is dispensable to the cell. It was found that this storage can be eliminated by growing the algae in a medium containing very little inorganic phosphate (see the "low" phosphate medium under "Preparation of cultures"). In Table III, the effects of washing on the internal P^{31} distribution in *Chlorella pyrenoidosa* grown in "high" and "low" phosphate media are compared.

Analyses for orthophosphate in the TCA extracts of Experiment 2 of Table III showed that the loss into the washes occurs at the expense of the TCA-soluble inorganic phosphate. With the Lowry-Lopez procedure (14), no labile phosphate esters of the acyl phosphate type could be detected in extracts of organisms grown in either type of medium. It is of interest that the final concentration of orthophosphate in the medium of full grown cultures on "high" phosphate is about 40 γ per ml., while the "low" phosphate medium is usually entirely depleted of phosphate. This difference parallels that observed by Mann (16) in growing cultures of *Aspergillus niger*. This mold grown in 0.2 to 0.5 per cent K_2HPO_4 media contains 1 to 2 per cent of its dry weight as phosphorus in contrast to 0.3 per cent when the medium contains only 0.02 per cent K_2HPO_4. In the latter case, the medium is depleted of phosphate rapidly. Mann has also demonstrated significant disparities in the metabolism of the two types of cultures.

TABLE III

P^{31} Distribution in Chlorella pyrenoidosa Grown in "Low" and "High" Phosphate Media

All cultures were 5 days old. The results are expressed in micrograms.

Medium Experiment No. Treatment	Low phosphate 1a Not washed	Low phosphate 1b Washed*	High phosphate 2a Not washed	High phosphate 2b Washed*
P in TCA extract	41.2	32.0	149.0	90.3
" " " residue	183.0	191.0	404.0	374.0
" " Wash 1		0		16.8
" " " 2				17.2
" " " 3				10.7
Total P	224.2	223.0	553.0	509.0

* The algae were washed with three 10 ml. portions of water at room temperature (approximately 0.2 ml. of wet volume algae in each series).

P^{32} Uptake—Typical results showing the effect of light on P^{32} uptake by *Chlorella* and *Scenedesmus* are given in Fig. 1, where specific activity in the TCA-insoluble residue is plotted as a function of time. Each point represents the average of duplicate determinations. In all of the experiments performed, there were no significant or consistent differences between light and dark samples with respect to P^{31} content of any of the fractions examined. The acid-soluble portion has as yet, however, not been examined in detail.

The turnover involving the insoluble fraction is distinctly greater in the light than in the dark. Moreover, the extent of this turnover is not directly proportional to the metabolic activity as indicated by gas exchange. For example, in the experiment of Fig. 1, *B*, the rates of O_2 absorption and production observed in control vessels were as follows: (*a*) In the absence of KCN, photosynthesis (corrected for respiration) = +129 microliters of O_2 per 30 minutes; respiration = −36.2 microliters of O_2 per 30 minutes. (*b*) In the presence of KCN, photosynthesis (corrected) = +37 microliters of O_2 per 30 minutes; respiration = −43.5 microliters of O_2 per 30 minutes.

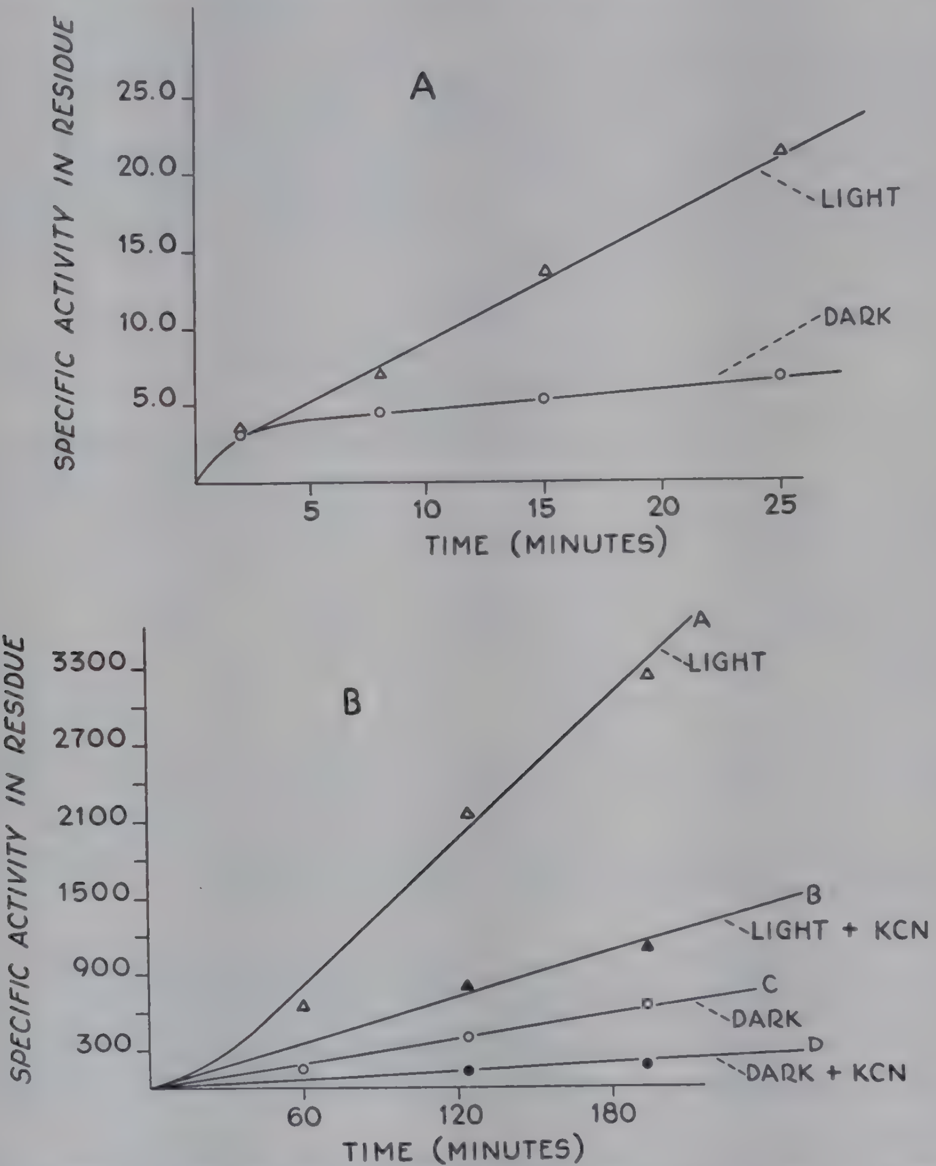


FIG. 1. Effect of illumination on P^{32} uptake by *Scenedesmus* D_3 and *Chlorella pyrenoidosa*. *A*, *Scenedesmus* D_3 in 0.1 M $NaHCO_3$ containing 0.16 mg. of orthophosphate as P per ml. and 0.76 mg. of $(NH_4)_2SO_4$ per ml.; temperature, 30°. *B*, *Chlorella pyrenoidosa* in Warburg Buffer 9; temperature, 22°; final concentration of KCN, 4.55×10^{-3} M.

Thus, although the endogenous respiration (dark) is stimulated slightly by KCN, the incorporation of P^{32} into the residue is markedly less in the presence of KCN than in its absence (Fig. 1, *B*, Curves C and D). The lack of correspondence between gas exchange and phosphate turnover can also be seen by comparing Curves B and D with the manometric data given for Fig. 1, *B*. This indicates that the differences observed cannot be attributed simply to a greater general metabolic activity during illumination.

Results of other uptake experiments with *Chlorella pyrenoidosa* are listed in Table IV.

The effect of illumination on phosphate turnover into the insoluble fraction in *Rhodospirillum rubrum* is more pronounced than that observed in the green algae. In the experiments summarized in Table V, the amount of CO_2 liberated by "autofermentation" in the dark was of the same order of magnitude as the CO_2 assimilated in the light. (This was true in Experiment B of Table V also, even though an attempt was made to deplete the endogenous reserves by aerating the cells in 0.05 per cent $NaHCO_3$ for 1.75 hours in the dark before use.) Considered with the P^{32} results, this again denotes the lack of a strict relation between extent of phosphate turnover and the over-all metabolic level as indicated by gas exchange. A large differential in the specific activity is seen even in the endogenous controls (Table V, Experiment B, Column *a*); apparently there was sufficient hydrogen donor present in the cells to permit extensive photosynthesis ("autoassimilation").

Delineation of the rôle played by phosphorylation in CO_2 reduction will depend to a great extent on a knowledge of the compounds actively engaged in the turnover of phosphate between the soluble reservoir and other portions of the cell. A preliminary attempt to identify the nature of these compounds was made with aliquots of the same bacterial suspension that was used for Experiment B in Table V. Results of a crude fractionation of the cells are presented in Table VI. After 160 minute exposures to light and dark, the cell suspensions were removed from the Warburg vessels, centrifuged, and the cells washed three times with 15 ml. portions of cold 0.85 per cent saline. Fractionations were then performed as described in the experimental section.

Recent work indicates that orthophosphate enters the "soluble" portion of the cell probably as a phosphoester and that organic phosphate compounds are subsequently derived from a reservoir in this fraction (17). This appears to be also true for photosynthetic microorganisms as evidenced by the fact that the highest degree of equilibration with exogenous phosphate is found in the soluble fraction (Tables IV and VI). Ordinarily, the extent of equilibration is easily determined in P^{32} uptake experiments;

in those reported here, the estimates are complicated by the phosphate exchange occurring between cells and medium. For example, in the experiments with *Chlorella pyrenoidosa* summarized in Table IV, Experiment A, the external specific activity at the end of the experiment was 71,700 counts per minute per microgram of P^{31}, while the specific activity of the added

TABLE IV

P^{32} Uptake by Chlorella pyrenoidosa in Warburg Buffer 9

Experiment A. 5 day-old culture grown in "high" phosphate medium; temperature, 20°

Time	Fraction	Light		Dark	
		Counts per min. per γ P^{31}	Per cent equilibration*	Counts per min. per γ P^{31}	Per cent equilibration*
min.					
118	Whole cells†	1560	2.36	524	0.76
		1825		560	
	TCA-soluble phosphate (calculated)	6320	8.8	2120	2.96
	TCA residue	406	0.57	131	0.17
		404		111	
273	" "	2120	2.88	368	0.51
		2000			

Experiment B. 4 day-old cultures grown in "low" and "high" phosphate media; duration of experiment, 165 min.; temperature, 25°

	Origin of culture			
	High phosphate		Low phosphate	
	Light	Dark	Light	Dark
P^{32} in TCA residue, *counts per min. per γ*	298.0	89.0	2490.0	885.0
	266.0	106.0	2320.0	850.0
P^{31} in residue, γ	50.3	50.5	41.0	36.6
	52.0	53.8	41.9	41.7
Total P^{31}, γ	242.6‡		63.4	
O_2 per 30 min., *microliters*	+163.4§	−28.0	+158.6§	−20.1

$$* \quad \frac{\text{Counts per min. per microgram } P^{31}}{\text{Counts per min. per microgram } P^{31} \text{ in external medium } (= 71{,}700)} \times 100.$$

† Cells washed three times with 10 ml. portions of water before measurement.

‡ 71 γ were found to be in the suspension liquid as orthophosphate (only 2.2 γ in the low phosphate experiment).

§ Corrected for respiration.

tracer was 1,120,000. Analyses for P^{31} showed that the external orthophosphate, initially from the tracer alone, had been diluted with unlabeled orthophosphate released from the cells. In this particular case, the final external specific activities were identical in light and dark samples.

The extent of equilibration in the various fractions of *Rhodospirillum rubrum* (Table VI) is more difficult to determine. Although the culture was grown in a medium with only a moderate concentration of inorganic phosphate (~40 γ of P per ml.), a rather large amount of phosphate was released into the solution while the culture was being aerated prior to the experiment proper (767 γ per 40 ml.; after resuspension in 40 ml. of fresh 0.05 per cent $NaHCO_3$, 5 ml. aliquots of the suspension were used for the experiments reported in Table VI). The tracer added had a specific activity of about 66,000 counts per minute per microgram of P^{31}. Judging from the final values of the specific activities in the external medium (P^{32} supernatant liquid), even more internal phosphate was released during the course of the experiment. The activities observed in the washes represent very little P^{31} (<2 γ per 15 ml. in the first wash). If the per cent equilibration is calculated on the basis of the final specific activities in the supernatant liquid as 100 per cent, then the effect of light on the turnover is even more pronounced than is indicated by the ratios of the specific activities given in the last column of Table VI (light *versus* dark).

TABLE V

P^{32} Uptake by Rhodospirillum rubrum (SI) in 0.05 Per Cent $NaHCO_3$

Experiment A, 7 day culture grown in "high" phosphate medium (700 to 800 γ of P per ml.) with *n*-butanol as H donor. *n*-Butanol was used also as H donor for the uptake experiment. Gas phase, 5 per cent CO_2 in N_2; temperature 25°; duration of experiment, 230 minutes; total P^{31}, 297 γ.

Experiment B, 68 hour culture grown in "low" phosphate medium (~40 γ of P per ml.) with acetate as H donor. Sodium butyrate was used as the H donor for the experiment. In Column *a*, light and dark, no H donor was added. Gas phase, 5 per cent CO_2 in N_2; temperature, 30°; duration of experiment, 150 minutes (cells "dissimilated" by aeration prior to experiment); total P, 117 γ.

	Experiment A					Experiment B					
	Light		Dark			Light			Dark		
	(*a*)	(*b*)	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)
P^{32} in residue, *counts per min. per γ*	159	175	8	8	7	2310	3070	3110	356	299	276
P^{31} in residue, γ	154	142	141	148	145	94	91	96	88	90	88

At any rate, it may be concluded that the uptake and turnover are both much greater in the light than in the dark. Although the residue contained a large quantity of lipide phosphorus, this fraction showed very little turnover. The bulk of the difference is encountered in the KOH extract, which represents "total protein" phosphorus. In contrast to yeast (17),[2] the KOH residue contains no detectable P^{31}; the counts observed in this fraction may well be due to contamination from the KOH extract.

"Flow" Experiments—Interpretation of P^{32} uptake experiments is sometimes complicated by the fact that high specific activity tracers are employed. Under these conditions, rather large amounts of radioactivity are associated with quantities of P^{31} which are beyond the range of detection by the usual colorimetric procedures. Appearance of activity in the soluble and insoluble fractions, without a significant change in distribution of P^{31}, does not then necessarily mean that there is any turnover between these fractions; *i.e.*, independent routes may exist for the uptake of minute amounts of P^{31} into the various fractions of the cell. In experiments of this nature, it is desirable to test this possibility by means of the "flow" experiment.

TABLE VI

P^{32} Uptake and Distribution in Rhodospirillum rubrum (SI) during Photosynthesis and Autofermentation

Culture and conditions similar to those described for Experiment B, Table V.

	Light			Dark			$\frac{(3)}{(6)}$
	Counts per min (1)	P^{31} (2)	Counts per min. per γ P^{31} (3)	Counts per min. (4)	P^{31} (5)	Counts per min. per γ P^{31} (6)	
		γ			γ		
P^{32} supernatant	374,000	22.9	16,300	2,120,000	45.8	46,250	
Wash 1	27,200	0		86,300	0		
" 2	8,090	0		8,000	0		
" 3	7,500			5,880			
TCA extract	550,500	62.5	8,825	284,000	65.5	4,335	2.04
" residue							
Lipide fraction	24,600	125.4	196	8,900	125.1	71.3	2.75
KOH extract	1,355,000	295.5	4,585	102,025	289.5	353.0	13.00
" residue	1,860	0		375	0		
Total	2,348,750	506.3		2,615,480	525.9		

This may be conducted in one of two ways. First, the cells can be grown in the presence of P^{32} and then differentially labeled by suitable procedures. For example, with P^{32}-labeled yeast, fermentation of glucose in the presence of unlabeled phosphate causes a considerably greater diminution of the specific activity in the acid-soluble portion compared with the acid-insoluble fraction (18). With such yeast, the effect of any given conditions on the flow *between the two fractions* may be readily determined. With green algae or *Rhodospirillum rubrum*, the differential in specific activities can be established by the simple expedient of washing with solutions containing unlabeled phosphate as already described. This first method, however, is unsatisfactory with photosynthetic microorganisms because the amount of phosphate involved in the light-accelerated turnover is too small to affect the initial specific activities measurably (particularly, if the initial differential is not very great). It would be possible to observe a significant difference in at least one of the fractions if the P^{31} distribution were very disproportionate.

In the second method, unlabeled cells are exposed to P^{32} (30 to 50 microcuries per ml. of suspension) for a relatively short time and then washed thoroughly to remove occluded tracer. This procedure usually results in a relatively high specific activity in the soluble fraction, while that in the insoluble is quite low. The cells are then subjected to light and dark in a solution preferably containing no exogenous phosphate. Flow between the two cellular fractions can then be followed in a system uncomplicated by exchanges occurring between cells and medium. The results of three flow experiments performed by the second method are given in Table VII (in Experiment C, exogenous phosphate was present as indicated in the foot-note; all values are the averages of duplicate or triplicate determinations).

The specific activity in the soluble fraction is invariably higher than that in the insoluble. With shorter exposure times (Experiments B and C, Table VII), the initial differential between the two fractions is somewhat enhanced.[3] Under the influence of light, the specific activity in the extract fraction decreases significantly more than in the corresponding dark samples. This indicates that light stimulated the flow of low specific activity phosphate from the insoluble fraction into the soluble portion. The simultaneous flow in the reverse direction is evidenced by corresponding increases in the specific activity of the residue.

In Experiment C, Table VII, the stimulatory effect of light on the phosphate turnover is apparent from the specific activities observed in the residues. The extract values, on the other hand, show no corresponding changes. This seeming discrepancy is explained by the fact that the P^{32} activity in the extract was much greater than that in the residue at the start of the experiment. Since ~100 γ of the extract phosphours were due to exogenous unlabeled phosphate, it is clear that the true specific activity of the soluble phosphate in the algae was considerably greater than 2200. Thus, the flow of several micrograms of soluble phosphate into the residue could change the specific activity of the latter 100 per cent, while an identical flow of residue phosphate into the soluble fraction would change the specific activity of the extract phosphate by only a few per cent or less.

---

[3] Similar results are obtained when carbon dioxide fixation is studied with C^{14} as tracer (Allen, M. B., and Kamen, M. D., unpublished experiments). A discussion of the turnover in the carbon compounds relative to solubility is given in a forthcoming monograph of the Photosynthesis Symposium of the American Association for the Advancement of Science, December, 1947.

TABLE VII

Flow of P between Soluble and Insoluble Fractions in Chlorella pyrenoidosa

Experiment A, 3 day culture grown in "high" phosphate medium. Exposed to P^{32} in Warburg Buffer 9 under good illumination for 20 minutes at 21°. Washed cells resuspended in Warburg Buffer 9 at 23° for 120 minutes.

Experiment B, 2 day culture grown in "low" phosphate medium. Exposed to P^{32} in Warburg Buffer 9 in light for 1 minute at room temperature. Washed cells resuspended in Warburg Buffer 9 at 25° for 197 minutes.

Experiment C, 4 day culture grown in "high" phosphate medium (containing added Fe and trace elements). Exposed to P^{32} in complete medium in light for 2 minutes at room temperature. Washed cells resuspended in complete medium at 20° for times indicated. Approximately 100 γ of the quantities listed as TCA-extractable phosphate in this experiment were present as exogenous phosphate.

Experiment		TCA extract		TCA residue	
		Counts per min. per γ P^{31}	P^{31}	Counts per min. per γ P^{31}	P^{31}
			γ		γ
A	Zero time	9985	19.0	1705	68.1
	Dark	7810	17.3	2240	67.5
	Light	6890	17.3	2565	60.3
B	Zero time	1970	27.5	113	124.5
	Dark	1073	30.4	280	117.0
	Light	734	34.3	340	115.3
C	Zero time	2175	131.0	159	84.3
	Dark, 80 min.	2245	128.5	189	84.1
	" 150 "	2200	132.0	196	91.0
	Light, 80 min.	2290	124.8	237	85.5
	" 150 "	2055	127.0	297	84.5

Estimates of the amount of turnover occurring can be made from these data by calculations involving a number of assumptions. Such calculations indicate that a fairly large fraction of the soluble phosphorus is participating in the light-stimulated turnover. Since the compounds concerned are as yet unknown, the estimates are at best very rough approximations. As an illustration, simple isotope dilution calculations can be applied to Experiment A (Table VII) as follows:

Let a = the specific activity in the residue at zero time, b = the specific activity in the residue at the end of the experiment, x = the quantity of phosphate transferred (this is assumed to be the same in both directions), and 68 = the micrograms of P^{31} in the residue. Then, for the dark, $b/a = 1.32$. Also,

$$\frac{b}{a} = 1.32 = \frac{9985x + (68 - x)(1705)}{68\ (1705)}$$

From this equation, $x = 4.4\ \gamma$. Solving the analogous equation for the amount moved in the light gives $x = 7.1\ \gamma$. Similar calculations in which the data for the soluble fraction are used give about the same result. In connection with these estimates, one point should be emphasized here; *viz.*, that the ratio of final specific activities in a particular fraction after incubation in light and dark ((specific activity in the light)/(specific activity in the dark)) is not directly proportional to the difference in turnover. It may be remarked that a small fraction of the total phosphate of the cells is involved in turnover as evidenced by the low equilibration values and the flow calculations. This fact makes the interpretation of simple uptake experiments difficult, particularly in view of the large amounts of phosphate moved about by washing procedures, the results of which in turn are influenced by culture conditions.

Finally it is worthy of note that the data of Table IV, Experiment B, and Tables V and VII show no obvious fundamental differences in phosphate turnover between cells grown in "low" or "high" phosphate media.

DISCUSSION

Before discussing the implications of these experiments to photosynthetic mechanisms, it seems worth while to consider briefly phosphate nutrition and its effects on the physiology of microorganisms. The nutritional aspects of phosphate metabolism have not been systematically investigated, and our information is limited to incidental observations made during the course of experiments dealing with biochemical transformations. This is generally true for observations on nitrogen nutrition also. The results of Mann (16) with *Aspergillus niger* show that the concentration of phosphate used in the growth medium has a profound effect on (*a*) the rate of phosphate utilization, (*b*) phosphate content, (*c*) rapidity and extent of growth, (*d*) respiratory quotient, (*e*) nitrogen metabolism, (*f*) content of certain vitamins, and (*g*) glucose metabolism. Similarly, it has been found (19) that the inorganic nitrogen content of the medium influences the protein content and the enzymatic constitution of *Bacterium coli* to a great extent.

In photosynthetic microorganisms, the phosphate content (and distribution) also depends on the concentration of inorganic phosphate in the medium. With "high" phosphate concentration, excess phosphate appears to be stored, primarily in the TCA-extractable fraction. This phosphate seems to be dispensable to the cells and readily leaks out into water, saline, etc., even at low temperature. Numerous examples of phosphate leakage from cells to medium can be found in data reported for various types of bacteria. In *Staphylococcus aureus* and *Streptococcus hemolyticus*, both N and phosphate are released into the supernatant in appreciable quantity compared with the amount extractable with 5 per cent TCA (even at low temperature (20)). Relatively large amounts of both inorganic and organic phosphate are soon found in the supernatants when *Streptococcus faecalis* is suspended in saline in the absence of glucose (21). During endogenous respiration, resting cells of *Thiobacillus thiooxidans* release inorganic phosphate into the medium (4, 22). Leakage of phosphate explains the failure of thoroughly washed *Bacterium coli* to ferment glucose, since addition of inorganic phosphate restores the activity (15). The actual leakage from *Bacterium coli* is evident in the data given by Macfarlane (23). In this case, a young culture of the bacteria after two washings with saline showed a rapid release of inorganic phosphate (into saline) in the absence of glucose. An appreciable quantity of the original phosphate of *Trypanosoma equiperdum* is lost when the organisms are washed with saline (24).

Release of phosphate from yeast is reported to be almost negligible. The experiments of Hevesy and Zerahn (25) with yeast grown in P^{32} disclosed that only 1 to 2 per cent of the total P^{32} content is lost into inactive nutrient solution (at 20° over a period of 24 hours). Before performing the experiment, however, the yeast was washed thoroughly with nutrient medium (containing phosphate) to remove adhering radioactivity. It is possible that some leakage or exchange occurred during the washing. According to Reiner,[4] about 10 per cent of the total P^{31} of yeast is released into distilled water over a period of several hours at room temperature (after being washed twice with cold distilled water). It is interesting to note that other experiments of Hevesy and Zerahn (25) showed that yeast internally labeled with K^{42} loses a large fraction of its K into the medium.

Prior to the present work it does not appear that any success has attended efforts to demonstrate an unequivocal relation between illumination and phosphate esterification (2, 5, 6, 26). Emerson *et al.* (2) attempted to observe shifts in phosphate distribution in various fractions of *Chlorella pyrenoidosa* which might have been expected to result when cells were illuminated in the presence and absence of CO_2. They concluded that, with the possible exception of "Ba-insoluble resistant phosphate," no significant changes occurred which could be interpreted as supporting hypotheses that conversion of radiant to chemical energy was mediated by "high energy" phosphate bond formation. However, only the TCA-soluble phosphate was fractionated in these researches. Our data indicate that TCA-soluble phosphate composition is strongly dependent on culture and washing conditions and procedures and thus it is difficult to assess the relevance of the distributions in P^{31} reported by Emerson *et al.* An attempt to use simple uptake of P^{32} as a definitive test for the existence of light coupling to phosphate esterification has recently been reported by Aronoff and Calvin (5). No light stimulation of phosphate pick-up was observed. It is evident, however, from our previous discussion of complications in the interpretation of simple uptake experiments and from the effects noted in our researches that the demonstration of phosphorylative mechanisms cannot be established without close attention to effects attendant on non-equilibration, washing procedures, and previous history of the cultures. One other point is worthy of comment. It has been found that in intact yeast fermenting glucose in the presence of fluoride no acyl phosphate can be detected.[5] Hence even under conditions in which a conversion of inorganic to acyl phosphate is reasonably certain to be occurring during metabolism in intact cells, it may be impossible with present analytical methods to demonstrate such phosphorylation.

---

[4] Reiner, J. M., private communication.

Arguments which have been presented against the notion of a phosphorylation mediated "directly" by light are based on the absence of any photochemical model reactions in which there occurs an efficient parceling of the energy of a single quantum into many smaller quanta 6, 26).[6] It should be noted that this type of objection can be used against *any* theory involving production and storage of energy in energy-rich bonds.

It is unnecessary to dwell upon the analogies which have been made between photosynthetic and chemosynthetic CO_2 reduction (27, 28). These may be paraphrased by the statement that mechanisms for CO_2 fixation are similar in both types of metabolism, but the ultimate energy source is different. Some evidence exists from the experiments of Vogler and coworkers (4, 27) that there may be mediation of phosphate bond formation by sulfur oxidation uncoupled from CO_2 uptake in *Thiobacillus thiooxidans*. The results obtained depended on the determination of inorganic phosphate in the medium during alternate periods of oxidation and CO_2 uptake. The assumption required was that disappearance of phosphate from the medium was synonymous with the formation of phosphate esters. It appears from our washing data that this assumption may well require more experimental justification. Further, an unexplained release of phosphate during endogenous metabolism of the organisms used by Vogler *et al.* complicates interpretation of the conclusions reached. Other difficulties, such as a very high ratio of CO_2 "fixed" to phosphate "esterified," uncertainty as to the state of reduction of the CO_2 taken up, etc. (6), would seem to indicate the desirability for additional experimental evidence bearing on the relation of phosphate esterification to CO_2 reduction. It is of interest to record the recent observations of McElroy (29) who has found that extracts from firefly lanterns can be prepared which in the presence of oxygen exhibit an increase in bioluminescence upon the addition of adenosine triphosphate. In this process, one observes a release of light quanta concomitant with oxidation, analogous to a "reverse photosynthesis." It appears that a phosphorylative mechanism is involved in absorption of oxygen and production of light. However, no evidence exists at present that this effect of "high energy" phosphate is direct.

---

[5] Spiegelman, S., private communication.

[6] Franck, J., private communication.

The light-induced phosphate turnover may be useful in monitoring activity of extracts prepared from photosynthetic organisms. It may be supposed that extracts which simulate the behavior of intact cells with respect to stimulation of phosphate turnover by light may be good test systems for analysis of components required in synthetic mechanisms.

SUMMARY

1. The uptake and turnover of phosphate in various cell fractions as influenced by light has been investigated by the use of two species of Chlorococcales (*Chlorella pyrenoidosa*, *Scenedesmus* D_3) and a species of Athiorhodaceae (*Rhodospirillum rubrum*).

2. It is shown that the gross phosphate distribution is altered appreciably by experimental conditions (culture media, washing procedures) prior to analysis of the organisms. In particular it is found that organisms grown in media with the phosphate composition usually recommended for optimal growth contain excess inorganic or highly labile phosphate which is easily removed by washing, while organisms grown in media of low phosphate content do not contain such washable phosphate. The uncertainty introduced into simple uptake experiments by these phenomena is discussed.

3. The phosphate uptake in the light is considerably greater than in the dark for all the organisms examined. Because of the uncertainties associated with the trichloroacetic acid-soluble phosphate, uptake data are usually referred to TCA-insoluble phosphate. The manometric data obtained with algae with KCN as an inhibitor of photosynthesis show that the uptake of phosphate is not directly related to the respiratory activity as measured by CO_2 evolution. In general no rigid proportionality appears to exist between phosphate uptake and the over-all metabolic level.

4. The present studies, in which internally labeled organisms are em-

ployed, indicate that cellular phosphate turnover and equilibration with exogenous phosphate are mediated by a small fraction of the soluble cellular phosphate. In experiments on flow of phosphate between insoluble and soluble cell fractions, it is found that light stimulates phosphate turnover.

5. The results obtained are discussed as well as those from other studies in the literature. It appears from these studies that ester phosphate may be formed as a result of light absorption, but that there is no experimental evidence which can decide whether such an esterification is, or is not, directly coupled with light absorption.

Addendum—A detailed description of the experiments of Aronoff and Calvin has appeared recently (*Plant Physiol.*, **23**, 351 (1948)). These workers used an indirect method for determination of inorganic and organic fractions of phosphate (P^{32}) taken up. Apparently the P^{31} contents of these fractions were not measured in any instance and consequently no data on specific activities are available. Since P^{31} contents were assumed from values given in the literature by other workers using different algae, the significance of the calculations of Aronoff and Calvin relating phosphate uptake and turnover to light and dark metabolism cannot be readily assessed.

BIBLIOGRAPHY

1. Ruben, S., *J. Am. Chem. Soc.*, **65**, 279 (1943).
2. Emerson, R. L., Stauffer, J. F., and Umbreit, W. W., *Am. J. Bot.*, **31**, 107 (1944).
3. Lipmann, F., in Nord, F. F., Advances in enzymology and related subjects, New York, **6**, 231 (1946).
4. Vogler, K. G., and Umbreit, W. W., *J. Gen. Physiol.*, **26**, 157 (1942).
5. Aronoff, S., and Calvin, M., Abstract of paper MDDC-1589, in Abstracts of declassified documents, **11**, No. 3, U. S. Atomic Energy Commission, Feb., 1948.
6. Rabinowitch, E. I., Photosynthesis and related processes, New York, **1**, (1945).
7. Gaffron, H., *Biochem. Z.*, **292**, 241 (1937).
8. Arnon, D. I., *Science*, **92**, 264 (1940).
9. van Niel, C. B., *Bact. Rev.*, **8**, 1 (1944).
10. Foster, J. W., *J. Gen. Physiol.*, **24**, 123 (1940).
11. Gaffron, H., *Biochem. Z.*, **275**, 301 (1935).
12. Lohmann, K., and Jendrassik, L., *Biochem. Z.*, **178**, 419 (1926).
13. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
14. Lowry, O. H., and Lopez, J. A., *J. Biol. Chem.*, **162**, 421 (1946).
15. Gale, E. F., The chemical activities of bacteria, Cambridge (1947).
16. Mann, T., *Biochem. J.*, **38**, 339 (1944).
17. Spiegelman, S., and Kamen, M. D., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **12**, 211 (1947).
18. Spiegelman, S., and Kamen, M. D., *Science*, **104**, 581 (1946).
19. Virtanen, A. I., and De Ley, J., *Arch. Biochem.*, **16**, 169 (1948).
20. Hotchkiss, R. D., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **4**, 153 (1944).
21. O'Kane, D. J., and Umbreit, W. W., *J. Biol. Chem.*, **142**, 25 (1942).
22. LePage, G. A., *Arch. Biochem.*, **1**, 255 (1942–43).
23. Macfarlane, M. G., *Biochem. J.*, **33**, 565 (1939).

24. Moraczewski, S. A., and Kelsey, F. E., *J. Infect. Dis.*, **82**, 45 (1948).
25. Hevesy, G., and Zerahn, K., *Acta radiol.*, **27**, 316 (1946).
26. Gaffron, H., in Green, D. E., Currents in biochemical research. New York, 25 (1946).
27. Umbreit, W. W., *Bact. Rev.*, **11**, 157 (1947).
28. van Niel, C. B., *Physiol. Rev.*, **23**, 338 (1943).
29. McElroy, W. D., *Proc. Nat. Acad. Sc.*, **33**, 342 (1947).

HEMOGLOBIN SYNTHESIS FROM GLYCINE LABELED WITH RADIOACTIVE CARBON IN ITS α-CARBON ATOM*

By KURT I. ALTMAN, G. W. CASARETT, R. E. MASTERS, T. R. NOONAN, AND K. SALOMON

(From the Department of Radiation Biology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York)

(Received for publication, May 21, 1948)

Important progress in our knowledge concerning the biological precursors of hemin was made when Bloch and Rittenberg (1) presented evidence that significant amounts of deuterium are incorporated into the hemin of rats fed deuterioacetic acid. Further advances in this field were made by Shemin and Rittenberg (2–4), who showed that glycine labeled with N^{15} is utilized as a nitrogenous precursor of hemin, whereas other possible amino acids, such as glutamic acid, proline, and leucine, do not act in a similar fashion and contribute only in an indirect way to hemin N by enriching the N^{15} concentration of the body. It has been reported recently that hemin synthesis from glycine occurs *in vitro* in the presence of avian blood (5) and with blood from patients with sickle cell anemia (6). It is of considerable interest for an understanding of the mechanism of hemin synthesis to determine whether the α-carbon atom of glycine is also incorporated in hemin.

Experiments demonstrating the participation of the α-carbon atom of glycine in the synthesis of hemin are reported in this paper. A preliminary report has been published elsewhere (7).

EXPERIMENTAL

In order to show that the methylene carbon atom of glycine is incorporated in hemin, two groups of animals were studied, one group consisting of four normal adult rats, and one group consisting of three adult rats which had been rendered anemic by previous phenylhydrazine feeding and which were responding to this anemia by increased hemoglobin production, as evidenced by an increased reticulocyte count.

These rats were fed methylene-labeled glycine ($C^{14}H_2NH_2COOH$)[1] of a specific activity of 1.83 microcuries per mg. in a single dose in aqueous solution by stomach tube. The total dose administered in this way was 2 microcuries, *i.e.* approximately 1 microcurie per 100 gm. of body weight.

---

* This work was performed under contract No. W-7401-eng-49 for the Atomic Energy Project at The University of Rochester.

[1] This preparation of glycine was synthesized by Dr. R. Ostwald and kindly made available to us through the courtesy of Dr. B. M. Tolbert of the Radiation Laboratory of the University of California.

At varying intervals after the glycine feeding, these animals were anesthetized with sodium pentobarbital and bled as completely as possible from the carotid artery by means of the technique of Buchwald and Hudson (8). This technique was modified first by the injection of a solution of sodium heparin into the femoral vein prior to bleeding from the carotid artery, and second by perfusion with physiological saline solution through the inferior vena cava, this perfusion being continued until the animal died. These modifications were adopted in order to secure maximum yields of hemoglobin and in order to free the organs of contained blood.

Hemoglobin was isolated by crystallization according to the method of Warburg and Reid (9). Crystalline hemin was prepared as described by Nencki and Zaleski (10). Globin was isolated according to Anson and Mirsky (11). Such globin preparations were carefully washed with acetone and ether and were found by spectroscopic analysis to contain no bound hemin.

Method of Analysis—A method developed by Bale and Masters[2] was employed for the determination of C^{14} activity. This method involved the conversion of organic material to carbon dioxide by the wet digestion method of Van Slyke and Folch (12). The CO_2 thus produced was then introduced into a 1200 ml. ionization chamber, together with inert CO_2, to produce a total pressure of 1 atmosphere. The ionization current was read through the use of a ballistic vane electrometer, a version of the dynamic condenser electrometer developed at The University of Rochester. The background ionization current is equivalent to approximately 200 C^{14} disintegrations per minute. Activities of this order are read to ± 10 per cent accuracy and net activities above twice the background to an accuracy of approximately ± 3 per cent.

Results

Glycine Feeding of Normal Rats—Data obtained from the feeding of methylene-labeled glycine to normal, untreated rats are presented in Table I, which shows the activities of hemin, globin, and hemoglobin, each of which was analyzed separately for C^{14} activity.

A comparison of the C^{14} content of globin with that of hemin is made for the following reasons: (1) a comparison of the respective C^{14} activity of hemin and globin would reveal whether the incorporation of C^{14} in hemin was due to the specific precursor activity of the α-carbon atom of glycine, or whether C^{14} incorporation in hemin was due to a non-specific enrichment of the C^{14} concentration of the body available for the synthesis of proteins and other large molecular components of the tissues; (2) data available on the C^{14} content of globin would permit the establishment of hemin-globin ratios (shown in the last column of Table I). This ratio may be considered an index of the C^{14} partition between hemin and globin and might possibly show measurable variations under pathological conditions, such as anemia, etc.

---

[2] Bale, W. F., and Masters, R. E., unpublished method.

The C^{14} content of the hemoglobin found checked fairly well with the theoretical value calculated from the C^{14} content of hemin and globin determined independently. In making this calculation a molecular weight of 68,000 was assumed for hemoglobin (13), and it was also assumed that 1 molecule of globin is capable of combining with 4 molecules of hemin (14). These theoretical values for hemoglobin are included in Tables I and II. The exact reason for the discrepancies is not apparent, although the most likely cause for this disagreement is contamination of hemoglobin or globin samples. In both groups of animals, one of the calculated hemoglobin values deviates markedly from the value found, in each case the measured value being lower than the calculated value. It is conceivable that a low activity contaminant, *e.g.* stroma, precipitated with hemoglobin, thus bringing about the observed disagreement between the calculated and measured values.

TABLE I

C^{14} Activity of Hemin, Globin, and Hemoglobin in Normal Rats after Feeding $C^{14}H_2NH_2COOH$

	C^{14} activity of hemin		C^{14} activity of globin		C^{14} activity of Hb				
Time after glycine feeding	Units, 10^4 disintegrations per min. per gm. hemin	Per cent dose in hemin*	Units, 10^4 disintegrations per min. per gm. globin	Per cent dose in globin*	Units, 10^4 disintegrations per min. per gm. Hb		Per cent dose in Hb*		Activity ratio, hemin-globin, gm. basis
					Measured	Calculated†	Measured	Calculated	
days									
1	25.2	0.49	2.84	1.42	3.54	3.69	1.83	1.91	8.9
6	31.0	0.66	4.61	2.57	2.93	5.65	1.69	3.23	6.7
8	18.3	0.29	2.20	0.86	2.69	2.82	1.10	1.15	8.3
8	17.4	0.46	2.31	1.56	2.04	2.87	1.43	2.02	7.5

* On the basis of total circulating blood.

† On the basis of hemin and globin measurements.

In Table I are also shown calculations of the percentage of the total dose of C^{14} incorporated in hemin, globin, and hemoglobin. These percentages were calculated from independent colorimetric hemoglobin determinations[3] before the animals were sacrificed or on the assumption that the hemoglobin of the rat represents 13.8 per cent of the total rat blood, which in turn amounts to 7 per cent of the total body weight. The value assumed for hemoglobin is based upon that published by Thewlis and Meyer (15) and checks with a small series of rats used in our laboratory.[4] In general, 0.5 per cent of the total dose of C^{14} administered was incorporated in hemin.

---

[3] Acid hematin was determined with the photoelectric colorimeter of Klett and Summerson.

Glycine Feeding of Phenylhydrazine-Treated Rats—Three rats were each given 24 mg. of phenylhydrazine hydrochloride by stomach tube on 2 alternate days, resulting in a depression of the erythrocyte count. These animals were then allowed to enter the recovery period, in the course of which the reticulocyte count reached a level of 15 to 20 per cent. When this level was reached, methylene-labeled glycine was administered.

Since these animals were synthesizing hemoglobin at a more rapid rate than normal rats, it is not surprising to find that the hemoglobin contained considerably higher concentrations of C^{14}. This is shown in Table II. It is interesting to note that the ratio of hemin to globin, expressed on a gm. to gm. basis, remains fairly constant and agrees quite well with that of the normal animals. This suggests that the partition of C^{14} in normal animals and in animals recovering from phenylhydrazine anemia varied within similar, narrow limits and was of the same order of magnitude. It cannot be said with certainty at this time whether the ratio of newly formed hemin to newly formed globin is constant under all conditions. There are indications, however, that under certain nutritional conditions[5] this hemin to globin ratio is altered extensively.

TABLE II

C^{14} Activity of Hemin, Globin, and Hemoglobin of Phenylhydrazine-Treated Rats after Feeding $C^{14}H_2NH_2COOH$

Time after glycine feeding	C^{14} activity of hemin, units, 10^4 disintegrations per min. per gm. hemin	C^{14} activity of globin, units, 10^4 disintegrations per min. per gm. globin	C^{14} activity of Hb, units, 10^4 disintegration per min. per gm. Hb		Activity ratio, hemin-globin, gm. basis
			Measured	Calculated*	
days					
1	52.2	7.18	3.42	8.9	7.3
4	58.5	8.24	10.9	10.2	7.1
5	160.0	16.6	23.5	22.1	9.6

* Calculated on the basis of hemin and globin measurements.

The significance of the hemin-globin ratios cannot be completely assessed at this time. However, the hemin-globin ratio is considered to reflect the rates at which hemin and globin are synthesized and the differences between these respective rates. The rates of incorporation of glycine in hemin and globin could be assessed by comparing the C^{14} activity per glycine residue incorporated in hemin and in globin. Such a comparison reveals that the C^{14} activity per glycine residue is approximately $1\frac{1}{2}$ times as high for hemin as for globin, suggesting that the rate of hemin synthesis is slightly faster than the rate of globin synthesis.

---

[4] For the blood volume, the assumed value is based on that cited by Griffith and Farris (16).

[5] Unpublished experiments of the authors.

For the purpose of making an approximate preliminary calculation, it is assumed that 75 per cent of the total C^{14} activity of globin is present as glycine residues,[6] and that there are 50 glycine residues per mole of globin.[7] It is furthermore assumed that 4 glycine residues are incorporated in every molecule of newly formed hemin.

DISCUSSION

The experiments reported in this paper indicate that the methylene carbon atom of glycine is incorporated into the hemin molecule, and that glycine labeled with C^{14} in its α-carbon atom acts as a precursor of the tetrapyrrole structure of hemin. Because of the finding that the amount of C^{14} activity of hemin is significantly greater than that of globin, it may be concluded that the incorporation of glycine into hemin is a process which does not depend upon the enrichment of the C^{14} concentration of the body. Thus, it appears probable that the α-carbon atom of glycine is incorporated directly into the hemin molecule. The mechanism of this reaction is at present unknown.

Shemin and Rittenberg (4) have pointed out that glycine may participate in hemin synthesis in a manner analogous to a reaction recently described by Fischer and Fink (18) in which a pyrrole-like substance is formed as a result of the condensation of glycine and a β-ketoaldehyde. Such a concept is tenable even in view of the report that the carboxyl carbon atom of glycine is not incorporated in hemin (19).[8] If the Fischer and Fink reaction is operative in hemin synthesis, it must be assumed that the carboxyl group is removed at some point during the condensation reaction or after the formation of the pyrrole ring.

It appears more likely that the carboxyl group is removed after the pyrrole ring has been formed, since no enzyme system capable of decarboxylating glycine has so far been discovered, the possible exception being the fermentation of glycine by *Diplococcus glycinophilus* (20, 21). This point of view is also supported by the experiment of Lorber and Olsen (22) who reported that no appreciable decarboxylation of glycine, labeled with C^{13} in the carboxyl group, takes place in isolated mammalian heart preparations. Furthermore, the hemin of rats to which $CaC^{14}O_3$ has been administered contains significant amounts of C^{14} (23). Thus, one would expect that, if carboxyl-labeled glycine were decarboxylated, the CO_2 thus formed could contribute to hemin synthesis, provided that its concentration was high enough.

---

[6] It is quite conceivable that the entire C^{14} activity of globin does not reside in the glycine residues, but that the C^{14} activity of other amino acids also contributes to the C^{14} activity of globin.

[7] The glycine content of rat hemoglobin is assumed to be analogous to that of horse hemoglobin as determined by Shemin and Foster (17).

[8] Radin, N., Rittenberg, D., and Shemin, D., personal communication.

Indirect evidence that the carbon-nitrogen bond in glycine remains intact is derived from the observation of Shemin and Rittenberg (3) that N^{15}-containing ammonium citrate fed to rats is not incorporated into hemin. These experiments also suggest that the oxidative deamination described by Ratner, Nocito, and Green (24) for a variety of tissues does not play a significant rôle as far as glycine utilization for hemin synthesis is concerned.

Suitable methods for the degradation of the hemin molecule are as yet not available. For this reason it has not been possible to determine the distribution of C^{14} in the tetrapyrrole structure of hemin. Attempts to develop such methods are now in progress in this laboratory.

SUMMARY

1. It has been shown that α-carbon-labeled glycine is incorporated into hemin and globin of normal and phenylhydrazine-treated animals, and that the hemoglobin of phenylhydrazine-treated animals contains higher concentrations of C^{14} activity than that of normal animals.

2. The data presented suggest that the partition of C^{14} activity between hemin and globin is essentially the same in normal animals and in animals recovering from an anemia produced by phenylhydrazine feeding.

The authors wish to express their thanks to Dr. William F. Bale for his continuing interest in this investigation.

BIBLIOGRAPHY

1. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **159,** 45 (1945).
2. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **159,** 567 (1945).
3. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **166,** 621 (1946).
4. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **166,** 627 (1946).
5. Shemin, D., London, I. M., and Rittenberg, D., *J. Biol. Chem.*, **173,** 799 (1948).
6. London, I. M., Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **173,** 797 (1948).
7. Altman, K. I., Casarett, G. W., Masters, R. E., Noonan, T. R., and Salomon, K., *Federation Proc.*, **7,** 2 (1948).
8. Buchwald, K. W., and Hudson, L., *Endocrinology*, **35,** 73 (1944).
9. Warburg, O., and Reid, A., *Biochem. Z.*, **242,** 147 (1931).
10. Nencki, M., and Zaleski, J., *Z. physiol. Chem.*, **30,** 384 (1900).

11. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, **13,** 469 (1930).
12. Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, **136,** 509 (1940).
13. Svedberg, T., and Fahraeus, R., *J. Am. Chem. Soc.*, **48,** 430 (1926).
14. Adair, G. S., *Proc. Roy. Soc. London, Series A*, **108,** 627 (1925); **109,** 292 (1925).
15. Thewlis, E. W., and Meyer, O. O., *Anat. Rec.*, **82,** 115 (1942).
16. Griffith, J. Q., Jr., and Farris, E. J., The rat in laboratory investigation, Philadelphia, 358 (1942).
17. Shemin, D., and Foster, G. L., *Ann. New York Acad. Sc.*, **47,** 119 (1946).
18. Fischer, H., and Fink, E., *Z. physiol. Chem.*, **280,** 123 (1944).
19. Grinstein, M., Kamen, M. D., and Moore, C. V., *J. Biol. Chem.*, **174,** 767 (1948).
20. Cardon, B. P., and Barker, H. A., *Arch. Biochem.*, **12,** 165 (1947).
21. Barker, H. A., Volcani, B. E., and Cardon, B. P., *J. Biol. Chem.*, **173,** 803 (1948).
22. Lorber, V., and Olsen, N. S., *Proc. Soc. Exp. Biol. and Med.*, **61,** 227 (1946).
23. Armstrong, W. D., Schubert, J., and Lindenbaum, A., *Federation Proc.*, **7,** 143 (1948).
24. Ratner, S., Nocito, V., and Green, D. E., *J. Biol. Chem.*, **152,** 119 (1944).

THE EFFECT OF TESTOSTERONE AND METHYLTESTOSTERONE ON GUANIDOACETIC ACID, CREATINE, AND CREATININE IN PLASMA AND URINE*

By BLAINE H. LEVEDAHL AND LEO T. SAMUELS

(From the Department of Biochemistry, University of Utah School of Medicine, Salt Lake City)

(Received for publication, April 28, 1948)

Since the major route of creatine formation in the animal body appears to involve the intermediate formation of guanidoacetic acid, methylated steroids such as methyltestosterone could cause the repeatedly observed (1, 2) increase in production of creatine by one of three possible mechanisms: (*a*) by transferring methyl groups to guanidoacetic acid, (*b*) by increasing production of guanidoacetic acid, or (*c*) by catalyzing some other system of creatine synthesis.

Wilkins and Fleishmann (3) in 1945 were able to demonstrate that only the methylated derivatives of the C_{19} steroid nucleus were effective in producing creatinurea. Methyltestosterone, methylandrostanediol-3,17, and methylandrostene-5-diol-3,17 all caused creatinurea, while ethyltestosterone and a number of other non-methylated steroids were found to be inactive. As a result of this work the idea was proposed that methylated steroids may act as catalysts for the process of methylating guanidoacetic acid to creatine.

The work of Borsook and Dubnoff (4, 5), du Vigneaud *et al.* (6), and Block and Schoenheimer (7) has established the probable mechanism of creatine formation as follows: (*a*) guanidoacetic acid is formed in the kidney from glycine and arginine and (*b*) the guanidoacetic acid is methylated by methionine, principally in the liver. Borsook *et al.* (8) demonstrated that administration of arginine and glycine to man led to a marked increase in guanidoacetic acid in the urine. Simmonds and du Vigneaud (9) furnished indisputable proof that the methyl group of methionine gives rise to creatinine in man. It therefore appears that this proposed mechanism applies to man.

If the rôle of catalyst proposed by Wilkins and Fleishmann were correct, it would follow that the increase in creatine levels should bear certain relations to the levels of guanidoacetic acid. Two alternatives appear possible. In the first case methylation of guanidoacetic acid could be accelerated

---

* This investigation was supported by a grant from Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

without an increase in its production; if this were the mechanism, the guanidoacetic acid levels of the blood and urine should decrease in direct relation to the increase in the creatine levels. In the second instance, the increased removal of guanidoacetic acid from the system by methylation could lead to increased guanidoacetic acid synthesis; thus its level would remain approximately constant while the creatine levels increased.

The present work was initiated to determine the plausibility of the proposed catalyst mechanism. Accordingly, normal male and female subjects were given methyltestosterone orally and the guanidoacetic acid and creatine levels of both the blood and urine were determined over an extended time. If the proposed mechanism operated, the levels of the precursor should remain constant or decrease while creatine increased. Similarly, the creatine and guanidoacetic acid levels of the blood and urine have been determined for normal male subjects given testosterone by injection, by implantation, and by oral administration. This series has served to check the possibility that the steroid nucleus as such would influence the guanidoacetic acid levels.

Methods

Dogs, rabbits, guinea pigs, rats, pigs, and monkeys have failed to show creatinurea following methyltestosterone administration. Consequently, it was necessary to use humans for this study. The selected subjects were placed on low creatine diets. 48 hours later the first of three consecutive 24 hour urine samples was collected. Blood samples were drawn at the end of each 24 hour collection period. Following collections of urine and blood, patients were permitted to return to normal diets. These normal diets were maintained until 48 hours before the next collections when the low creatine diet was again employed.

Administration of methyltestosterone or testosterone was begun on the last day of the first collection period. In the case of methyltestosterone, one 10 mg. tablet was given before each meal and two before retiring, making a total of 50 mg. per day. In the series utilizing testosterone propionate one subject was given 50 mg. of testosterone propionate in peanut oil orally, two subjects were given intramuscular injections of 25 mg. of the same compound every 2 days, and one subject had four 150 mg. pellets of testosterone propionate implanted intramuscularly in the left infrascapular region.

The plasma and urine were analyzed for preformed and total creatinine (10). The method of Peters was altered by lengthening the autoclave period to 45 minutes to complete the conversion of creatine to creatinine at this altitude, and by diluting the urine specimens to constant volume rather than to constant specific gravity. This latter change was made only for convenience in the analysis, since variation in specific gravity was not large.

Guanidoacetic acid was estimated by the method of Dubnoff and Borsook (11). This method was modified slightly in that the samples were warmed to room temperature by rubbing the sample container between the hands for 1 minute before colorimetric readings were made. The creatinine and guanidoacetic acid determinations were run in duplicate on each sample.

TABLE I

Guanidoacetic Acid Recoveries with Permutit Separation (Average of Duplicate Determinations)

Test No.	Arginine added	Guanidoacetic acid added	Guanidoacetic acid recovered
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*
1	0	0.125	0.121
2	0	0.250	0.251
3	0	0.50	0.50
4	0	1.00	1.02
5	30	3.0	2.72
6	30	1.0	0.98
7	20	1.0	1.0
8	10	3.0	2.70
9	10	1.0	1.04
10	0	0	0

TABLE II

Creatinine Recoveries after Autoclaving with Guanidoacetic Acid (Average of Duplicate Determinations)

Test No.	Creatinine added	Guanidoacetic acid added	Creatinine recovered
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*
1	0.83	0	0.84
2	0.83	0.25	0.83
3	0.83	0.50	0.84
4	0.83	1.00	0.84

Results

Preliminary Measurements—Since some workers (12, 13) have experienced difficulty with the method for the determination of guanidoacetic acid as proposed by Dubnoff and Borsook, recovery measurements were made utilizing varying quantities of guanidoacetic acid and arginine. The results are given in Table I. These data show that satisfactory separation of the arginine and guanidoacetic acid was obtained with the permutit samples used. Further, added guanidoacetic acid could be recovered with a precision of approximately 5 per cent or better in the range of physiological concentrations.

If present in sufficient quantity, the anhydride of guanidoacetic acid, formed during autoclaving, could be an interfering substance in the creatine

TABLE III

Creatine, Creatinine, and Guanidoacetic Acid Levels in Plasma and Urine of Male and Female Subjects Given 50 mg. of Methyltestosterone Daily

Each value represents duplicate samples on 3 consecutive days.

	Days on experiment	Male			Females		
		M. J. B.	L. B.	B. H. L.	L. A.	M. H.	M. S.
Plasma creatine, *mg. per 100 cc.*	0				0.33	0.27	0.52
	13	0.45	0.35	0.37	0.44	0.40	0.61
	22*				0.69	0.83	1.05
	28	0.50	0.48	0.59			
	42†	0.80	0.61	0.74			
	56	0.49	0.51	0.33			
	72	0.57	0.42	0.42			
Urine creatine, *gm. per day*	0				0.046	0.032	0.057
	13	0.046	0.053	0.047	0.036	0.080	0.065
	22*				0.255	0.402	0.523
	28	0.047	0.048	0.053			
	42†	0.183	0.160	0.134			
	56	0.029	0.055	0.038			
	72	0.040	0.040	0.033			
Plasma guanidoacetic acid, *mg. per 100 cc.*	0	0.26	0.28	0.24	0.34	0.26	0.25
	13	0.24	0.27	0.26	0.46	0.34	0.54
	22*				0.45	0.43	0.45
	28	0.26	0.22	0.19			
	42†	0.42	0.40	0.38			
	56	0.39	0.25	0.27			
Urine, guanidoacetic acid, *gm. per day*	0	0.036	0.024	0.016	0.026	0.037	0.028
	13	0.073	0.041	0.046	0.051	0.105	0.067
	22*				0.118	0.272	0.175
	28	0.065	0.051	0.070			
	42†	0.114	0.085	0.114			
	56	0.059	0.051	0.039			
	72	0.061	0.040	0.034			
Plasma creatinine, *mg. per 100 cc.*	0				0.88	0.92	0.84
	13	1.37	1.34	1.36	1.02	1.03	1.05
	22*				1.00	1.04	1.05
	28	1.17	1.31	1.21			
	42†	1.19	1.41	1.40			
	56	1.26	1.42	1.47			
	72	1.41	1.50	1.50			

TABLE III—*Concluded*

	Days on experiment	Males			Females		
		M. J. B.	L. B.	B. H. L.	L. A.	M. H.	M. S.
Urine creatinine, *gm. per day*	0				0.907	1.182	1.143
	13	1.776	1.873	1.812	1.150	1.368	1.273
	22*				0.955	1.307	1.302
	28	1.763	1.934	1.967			
	42†	1.767	2.094	2.112			
	56	2.037	2.067	2.147			
	72	1.927	2.167	2.240			

* Methyltestosterone withdrawn from female subjects.

† Methyltestosterone withdrawn from male subjects.

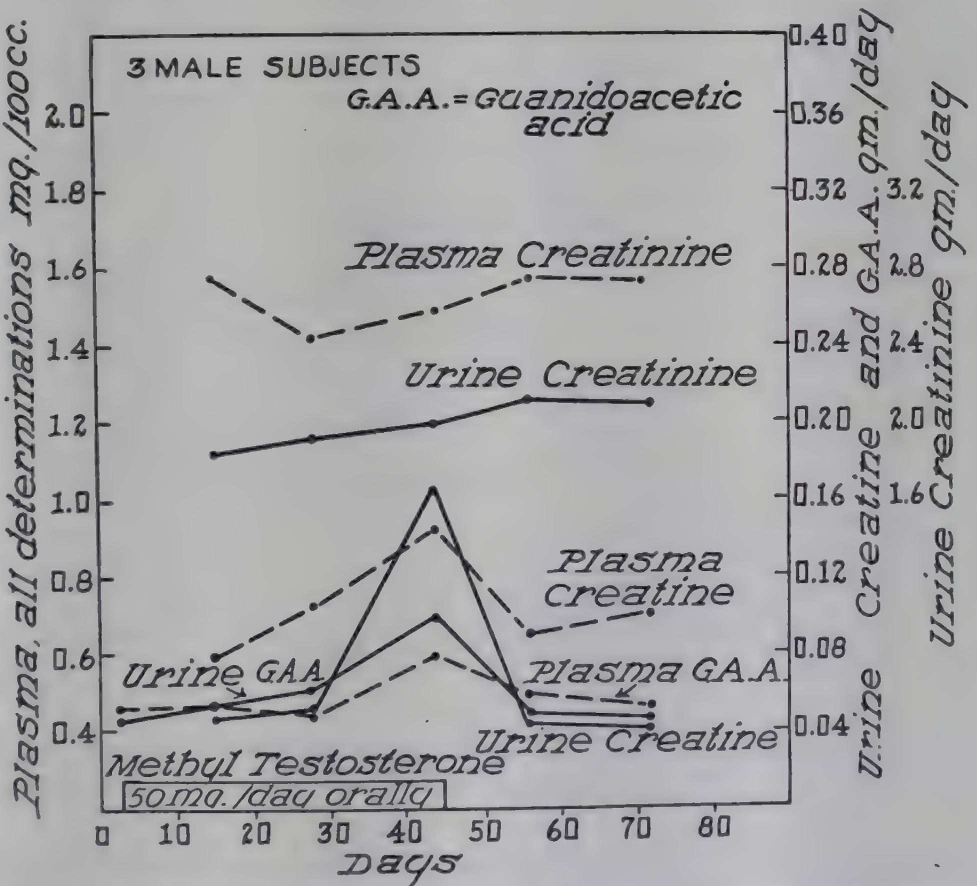


FIG. 1. Average guanidoacetic acid, creatine, and creatinine levels of plasma and urine for three male subjects given 50 mg. per day of methyltestosterone orally.

determination. To check this possibility creatinine determinations were made after 45 minutes autoclaving on samples containing varying amounts of guanidoacetic acid and creatinine. The results of these experiments are given in Table II. These data show that guanidoacetic acid in the quantities encountered in this work does not represent an interfering substance in the creatine determination. The N-methyl group of creatinine is known to prevent the interference of this compound in the determination of guanidoacetic acid (14).

Studies Involving Methyltestosterone—The plasma and urine levels of creatine and guanidoacetic acid were determined in three normal men and three normal women receiving methyltestosterone. The results are given in Table III, and the average results are shown in Figs. 1 and 2. In the cases involving the men 50 mg. of methyltestosterone were administered daily for 43 days. Significant increases in urine levels of creatine and guanidoacetic acid occurred after an initial delay of approximately 20 days. The plasma levels were significantly higher at 20 days than at 10 days. Original plasma levels were not determined in this series. 10 days following cessation of methyltestosterone administration, all levels had returned to normal.

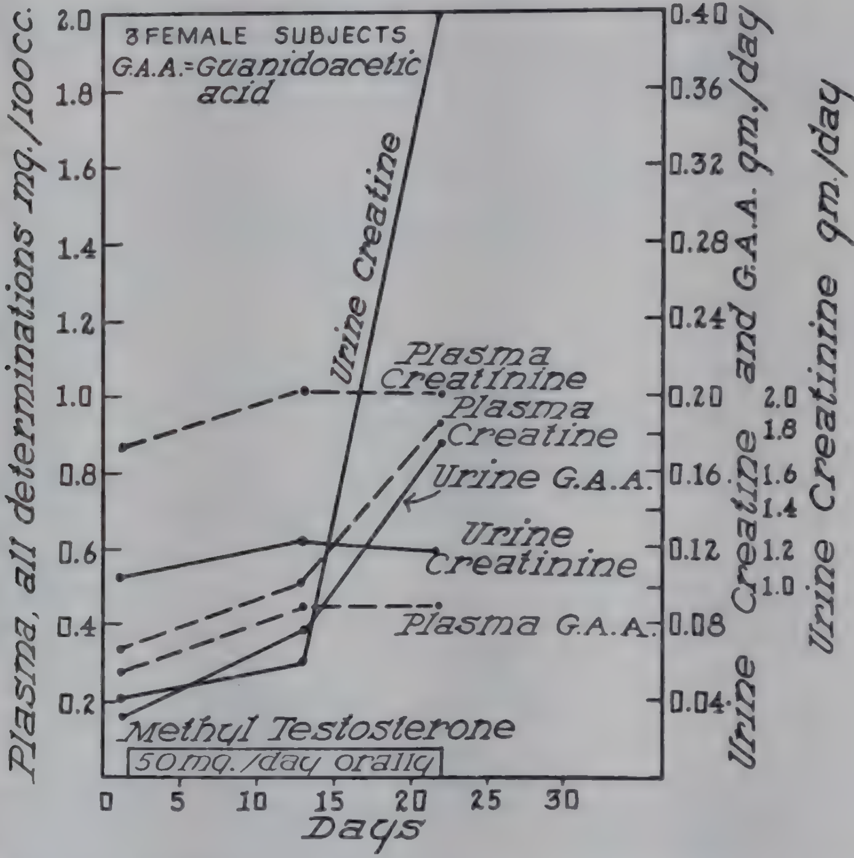


FIG. 2. Average guanidoacetic acid, creatine, and creatinine levels of plasma and urine for three female subjects given 50 mg. per day of methyltestosterone orally.

The female subjects received 50 mg. of methyltestosterone a day for

20 days. There was no significant delay before the elevation of the creatine and guanidoacetic acid levels of plasma. The urine guanidoacetic acid level also rose significantly, until the plasma level passed the renal threshold of 0.6 to 0.7 mg. per 100 cc. The values of all constituents except plasma guanidoacetic acid rose throughout the 20 day period of treatment. The leveling off of the plasma guanidoacetic acid at approximately 0.45 mg. per 100 cc. is thought to represent the maximal renal reabsorption level of this compound.

TABLE IV

Creatine, Creatinine, and Guanidoacetic Acid Levels in Male Subjects Given Testosterone Propionate

Each value represents duplicate samples on 3 consecutive days.

	Days on experiment	J. W.	I. K.	B. H. L.	M. J. B.
Plasma creatine, *mg. per 100 cc.*	0	0.37	0.34	0.29	0.37
	13	0.34	0.32	0.30	0.42
	22	0.28	0.26	0.28	0.34
	41	0.31	0.33	0.26	0.38
Urine creatine, *gm. per day*	0	0.033	0.025	0.027	0.030
	13	0.040	0.026	0.030	0.023
	22	0.035	0.015	0.005	0.010
	41	0	0	0	0
Plasma guanidoacetic acid, *mg. per 100 cc.*	0	0.44	0.34	0.30	0.27
	13	0.45	0.34	0.27	0.27
	22	0.55	0.32	0.30	0.31
	41	0.55	0.36	0.35	0.34
Urine guanidoacetic acid, *gm. per day*	0	0.048	0.052	0.036	0.057
	13	0.045	0.047	0.028	0.058
	22	0.051	0.042	0.033	0.058
	41	0.050	0.057	0.031	0.062
Plasma creatinine, *mg. per 100 cc.*	0	1.49	1.21	1.33	1.26
	13	1.52	1.33	1.62	1.57
	22	1.46	1.23	1.28	1.26
	41	1.50	1.27	1.27	1.35
Urine creatinine, *gm. per day*	0	1.470	1.180	1.847	1.350
	13	1.470	1.512	1.845	1.895
	22	1.567	1.678	2.030	1.981
	41	1.690	1.600	1.710	1.800

Studies Involving Testosterone Propionate—While the action of testosterone propionate on the creatine levels of normal subjects is well known, no information has been available on the guanidoacetic acid levels. Consequently, the plasma and urine levels of creatine and guanidoacetic acid were determined for four normal male patients receiving testosterone propionate as described. By using intramuscular injection, implantation, and oral administration of testosterone propionate, it was possible to eliminate the variable of route of administration from the measurements. The results of these experiments are given in Table IV and the average results shown in Fig. 3.

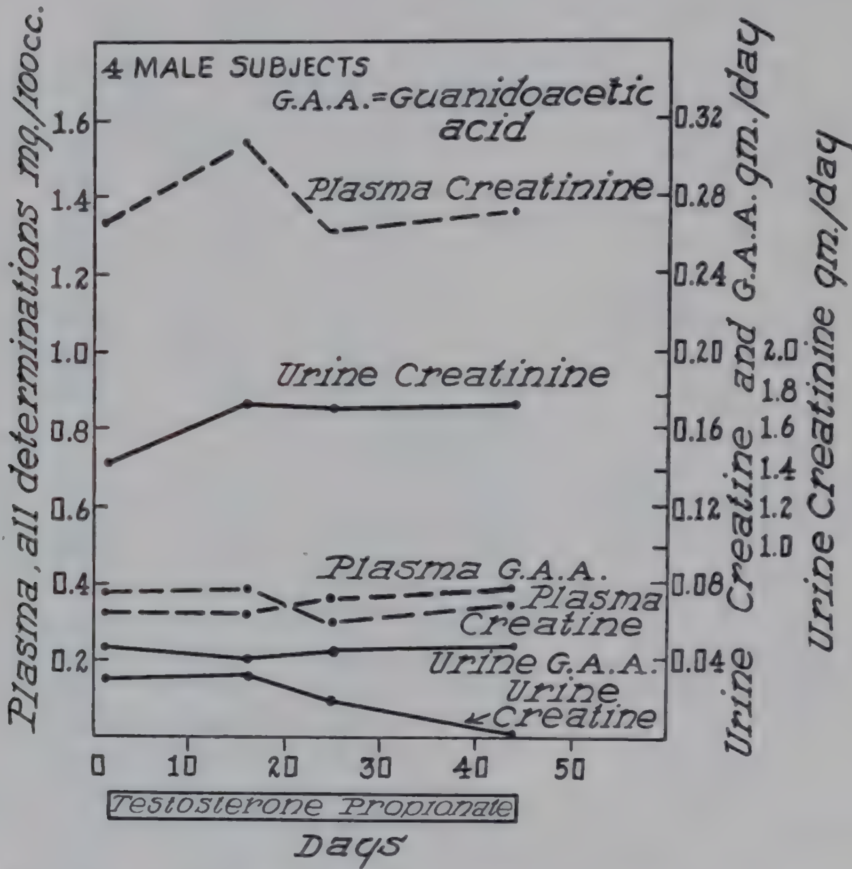


FIG. 3. Average guanidoacetic acid, creatine, and creatinine levels of plasma and urine for four male subjects given testosterone propionate by various routes of administration. The route of administration did not affect results.

These data show that there was no significant change in the plasma creatine and guanidoacetic acid levels and the urine guanidoacetic acid level during the course of the experiment. After an initial 10 day delay the urine creatine level decreased in all subjects regardless of the route of administration of the testosterone propionate. All other levels remained constant throughout the administration period.

DISCUSSION

The results of this study lead to the elimination of the previously proposed rôle of methyltestosterone in creatine metabolism. Since the guanidoacetic acid levels as well as the creatine levels are elevated following methyltestosterone administration, the rôle of a methyl-transferring agent previously suggested for methyltestosterone is no longer tenable. The elevation of the guanidoacetic acid levels of plasma and urine indicate that the methyltestosterone must act primarily at some stage prior to guanidoacetic acid methylation. The steroid nucleus as such was obviously not involved in the process of increased formation of guanidoacetic acid, since testosterone was ineffective.

The evidence of Samuels, Sellers, and McCaulay (15) that acute renal damage decreases or prevents the effect of methyltestosterone indicates that the kidney is the site of the increased formation of the guanidoacetic acid, apparently the primary effect of methyltestosterone in creatine metabolism. This is additional evidence that the order of synthesis demonstrated in rats is also that followed in the human. Additional statements of the action of methyltestosterone must await the elucidation of the particular system catalyzed by the methylated steroids.

SUMMARY

1. Guanidoacetic acid and creatine levels have been determined in normal male and female subjects who had received methyltestosterone. The guanidoacetic acid and creatine levels were found to increase in the same general fashion in both plasma and urine.
2. The possibility of the steroid nucleus *per se* bringing about increased guanidoacetic acid production has been eliminated.
3. Methyltestosterone affects some process associated with the synthesis of guanidoacetic acid, rather than its methylation alone.

BIBLIOGRAPHY

1. Wilkins, L., Fleishmann, W., and Howard, J. E., *Bull. Johns Hopkins Hosp.*, **69**, 493 (1941).
2. Samuels, L. T., Henschel, A. F., and Keys, A., *J. Clin. Endocrinol.*, **2**, 649 (1942).
3. Wilkins, L., and Fleishmann, W., *J. Clin. Invest.*, **24**, 21 (1945).
4. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **132**, 559 (1940).
5. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **134**, 635 (1940).
6. du Vigneaud, V., Chandler, J. P., Cohn, M., and Brown, G. B., *J. Biol. Chem.*, **134**, 787 (1940).
7. Block, K., and Schoenheimer, R., *J. Biol. Chem.*, **138**, 167 (1941).
8. Borsook, H., Dubnoff, J. W., Lilly, J. C., and Marriott, W., *J. Biol. Chem.*, **138**, 405 (1941).
9. Simmonds, S., and du Vigneaud, V., *J. Biol. Chem.*, **146**, 685 (1942).

10. Peters, J. H., *J. Biol. Chem.*, **146**, 179 (1942).
11. Dubnoff, J. W., and Borsook, H., *J. Biol. Chem.*, **138**, 381 (1941).
12. Sims, E. A. H., *J. Biol. Chem.*, **158**, 239 (1945).
13. Hoberman, H. D., *J. Biol. Chem.*, **167**, 721 (1947).
14. Poller, K., *Ber. chem. Ges.*, **59**, 1927 (1926).
15. Samuels, L. T., Sellers, D. M., and McCaulay, C. J., *J. Clin. Endocrinol.*, **6**, 655 (1946).

CHROMATOGRAPHY OF AMINO ACIDS ON STARCH COLUMNS. SEPARATION OF PHENYLALANINE, LEUCINE, ISOLEUCINE, METHIONINE, TYROSINE, AND VALINE

By WILLIAM H. STEIN and STANFORD MOORE

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, June 8, 1948)

In the past few years, several methods for the chromatographic fractionation of mixtures of amino acids have been introduced by Martin and Synge and their coworkers. Chromatography of the N-acetyl amino acids on silica gel columns has been employed by Martin and Synge (1, 2), and has been further studied by Tristram (3). The separation of the free amino acids by chromatography on paper, following its introduction by Consden, Gordon, and Martin (4), has found wide use. A third method, chromatography on starch columns, has been the subject of a note by Elsden and Synge (5), and was used by Synge (6) in qualitative experiments with partial hydrolysates of gramicidin.

It appeared that the starch column should possess characteristics rendering it the technique of choice for some types of problems. As Synge (6) pointed out, free amino acids and peptides may be chromatographed on starch, blocking of the amino group by acetylation being unnecessary. In addition, it was to be anticipated that mixtures should be fractionable in sufficient quantity with starch columns to permit subsequent examination of the components by conventional microchemical techniques. The procedure also appeared to be one which could be developed into a quantitative method for amino acid analysis.

The investigations described in this communication deal with developmental work on the chromatography of amino acids on starch columns and with specific methods for the separation and quantitative determination of phenylalanine, leucine, isoleucine, methionine, valine, and tyrosine in protein hydrolysates. Studies on the remaining common amino acids form the subject of a paper now in preparation.

In the fractionation of partial hydrolysates of gramicidin on starch columns, Synge (6) collected the effluent from the column in relatively large fractions and spot-tested each qualitatively with ninhydrin-impregnated paper. The volume of a fraction was adjusted to include substances within a given range of zone rates. Each fraction was worked up individually and its contents examined.

In the present investigations, the effluent has been collected in a regular

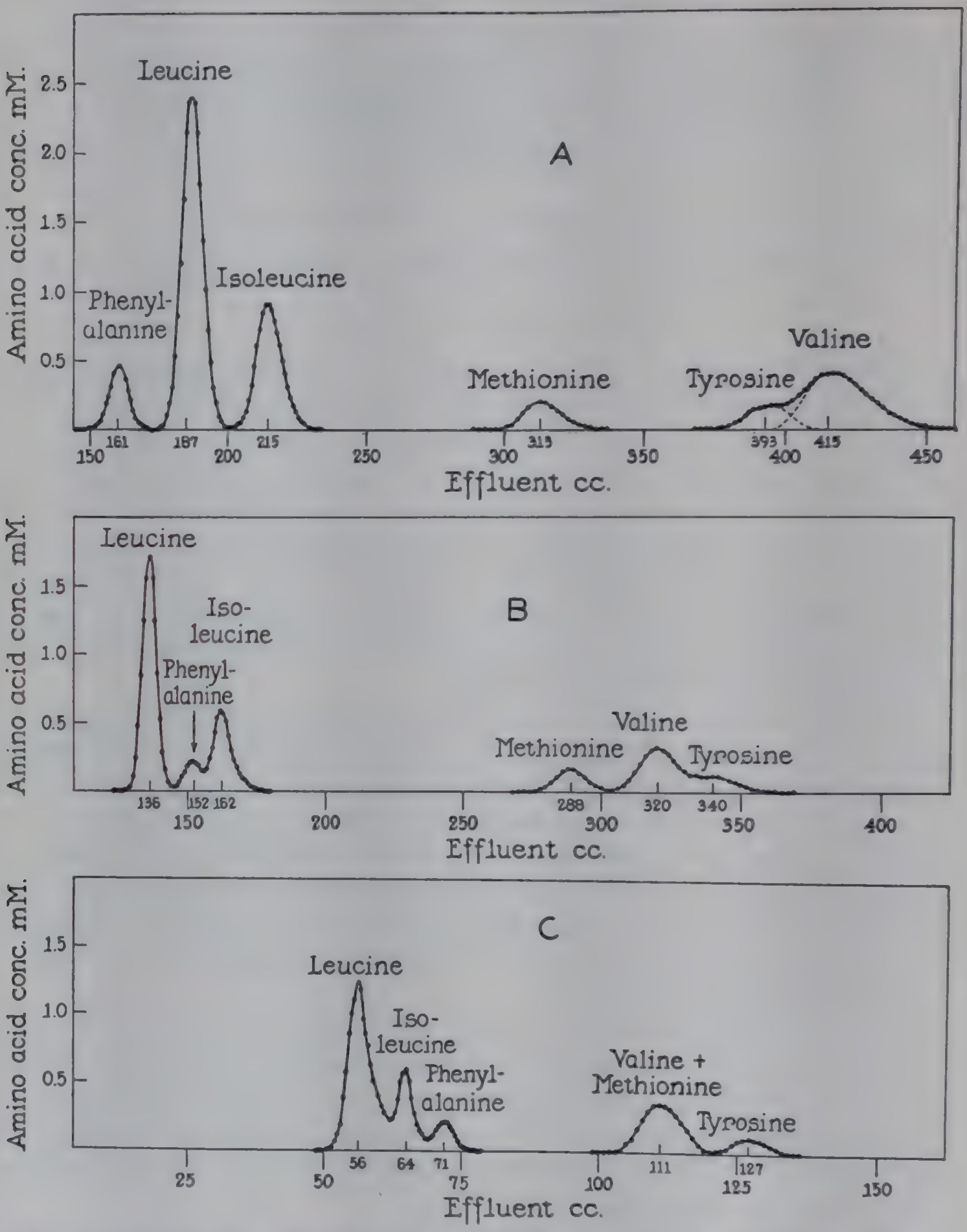


FIG. 1. Separation of amino acids from a synthetic mixture containing eighteen amino acids and NH_4Cl, corresponding in composition to an acid hydrolysate of β-lactoglobulin. Amino acid concentrations are given in leucine equivalents (11). *A*, solvent, 1:1:0.288 *n*-butanol-benzyl alcohol-water. Column, 52 gm. of starch (anhydrous); diameter, 1.9 cm.; height, 30 cm. Sample, 20 mg. of synthetic mixture. *B*, solvent, *n*-butanol-15 per cent water. Column, 55 gm. of starch (anhydrous); diameter, 1.9 cm.; height, 29 cm. Sample, 10 mg. of synthetic mixture. *C*, solvent, *n*-butanol-17 per cent 0.57 N HCl. Column, 55 gm. of starch (anhydrous); diameter, 1.9 cm.; height, 28 cm. Sample, 5 mg. of synthetic mixture.

series of small fractions of known volume, and the fractions have been analyzed quantitatively rather than qualitatively. The data thus obtained permit the construction of effluent concentration curves which reveal the detailed behavior and the full resolving power of the column. The effluent fractions have been collected with the aid of a specially constructed, fully automatic fraction-collecting machine, and the fractions have been analyzed quantitatively for amino acids by means of a photometric ninhydrin method developed for this purpose.

Effluent concentration curves showing the behavior of six amino acids in three solvents are given in Fig. 1. These amino acids are the first to emerge from a starch column when the sample fractionated consists of a known mixture of eighteen amino acids and ammonium chloride made up to simulate the composition of an acid hydrolysate of β-lactoglobulin (*cf.* data of Brand *et al.* (7)). The procedure employed to obtain curves of the type shown in Fig. 1 is given in the experimental section, followed by a discussion of the influence of variables on the process and by a summary of the results obtained with protein hydrolysates.

Procedure

Preparation of Starch Column—The chromatograph tubes used in these experiments were of the Zechmeister-Cholnoky type with ground joints and sintered glass plates.[1] The following directions are for the preparation of a 30 cm. starch column in a tube of 0.9 cm. inner diameter and 40 cm. in length. Direct proportionality factors can be used for columns of other dimensions. The columns were prepared and run in an air-conditioned room at 25° ± 0.5°. There have been no indications that a constant temperature room is essential. Recent experiments have indicated that satisfactory results can be obtained at room temperatures of 25° ± 5°.

The starch employed in this investigation was potato starch powder manufactured by Morningstar Nicol, Inc.[2] Different batches procured from the same manufacturer have given fully reproducible results. In order to obtain the correct tightness of column packing, the water content of the starch at the time the column is prepared must be carefully controlled. The moisture content of the air-dried product is determined by drying a sample to constant weight at 110° at atmospheric pressure. The moisture content of starch may vary with the atmospheric humidity, and should be checked periodically. An air-dried sample corresponding to 13.4 gm. of

---

[1] Purchased from the Scientific Glass Apparatus Company, Bloomfield, New Jersey, catalogue No. J-1664. A length of 40 cm. above the sintered plate must be specified for use with starch columns 30 cm. in height.

[2] Manufactured from white potatoes by Morningstar Nicol, Inc., New York, and purchased from Amend Drug and Chemical Company, Inc., New York.

anhydrous starch is weighed out. For the starch used in these experiments, the sample was suspended in 25 cc. of dry butanol in which enough water had been dissolved to bring the total water present to 30 per cent of the dry weight of the starch. For starch containing 20 per cent moisture, 16.8 gm. were weighed out and suspended in butanol containing 0.7 cc. of water. The starch is stirred thoroughly with a glass rod until a uniform suspension free from lumps is obtained. During this time the starch adsorbs most of the water present in the butanol. The suspension is poured into the upright chromatograph tube through a funnel possessing a tip bent to touch the side of the tube. In this manner, the suspension flows down the side of the tube without incorporation of air bubbles. For chromatograph tubes up to 2 cm. in diameter, a 20 cm. extension of glass tubing of the same diameter is attached to the top of the tube during the pouring process. This extension is necessary to accommodate the full volume of the slurry. The connection is secured glass to glass with rubber tubing. For tubes possessing a diameter larger than 2 cm., the columns can be packed in portions without an extension by first pouring two-thirds of the slurry and adding the remainder after the first portion has settled to constant height.

After the suspension has been poured into the tube, the column is placed under an air pressure of 5 to 7 cm. of mercury.[3] The starch settles slowly over a period of 1 to 3 hours. If the moisture content of the starch has been correctly adjusted, a sharp settling line can be seen to move steadily up the tube when the column is illuminated from behind with a strong light. When the starch has packed to a constant height, the extension tube, if one was used, is removed and the butanol remaining on the surface of the column is withdrawn with a pipette attached to a rubber bulb. The solvent with which the chromatogram is to be run is added carefully to the top of the column without disturbing the surface of the starch. The tube is filled to within 5 cm. of the top. A 125 cc. separatory funnel filled with solvent is attached to the column by a micro rubber stopper (Arthur H. Thomas Company, catalogue No. 8823-A) through which the constricted tip extends about 2 cm. An air lock is thus formed between the solvent and the stopper in order to avoid contact of the liquid with the rubber. A pressure of 8 cm. of mercury is applied to the top of the separatory funnel and maintained until about 50 cc. of solvent have passed through the column. This procedure, which usually requires about 36 hours, permits the starch to adsorb enough water to become equilibrated with the wet organic solvent. The resulting swelling of the starch may cause the surface of the column to rise 1 to 2 cm. If butanol-benzyl alcohol solvent mixtures have been employed, the column will gradually become translucent during the equilibration process.

---

[3] For routine use, reducing valves are installed on the compressed air lines. Air, filtered through a ½ inch Logan aridifier (Crane and Company, 47–30 29th Street, Long Island City, New York) is drawn at 70 pounds pressure through a reducing valve type R-79 (Linde Air Products Company, 205 East 42nd Street, New York). The outlet gage on the valve is replaced by a test gage reading from 0 to 25 cm. of mercury, 2½ inches in diameter, procured from the Factory Products Company, 161 Meserole Avenue, Brooklyn 22. A T-tube with a rubber tube and screw clamp is placed in the line to the chromatograph tube to provide a constant "bleeder."

After the starch columns have become fully equilibrated, they are operated under a pressure of 15 cm. of mercury. Pressure in excess of 8 cm. during the equilibration period, or of 15 cm. during operation, may cause the generation of air bubbles in the lower half of the column, and, therefore, should not be used. The presence of a small number of air bubbles within a few cm. of the sintered plate is frequently noted, and does not interfere with the results.

The degree of tightness of the column packing must be checked by measurement of the flow rate after equilibration with butanol-water or butanol-benzyl alcohol-water. A satisfactory column, 0.9 cm. in diameter and 30 cm. in height, should possess a flow rate of 1.25 to 1.50 cc. per hour at 15 cm. of mercury pressure (2.0 to 2.4 cc. per hour per sq. cm. of cross-sectional area on a column 30 cm. in height). Preparations of potato starch from different sources may vary in average particle size, which can affect the amount of water which should be present when the column is poured. In general, it can be suggested that if too fast or too slow a flow is obtained, the water content at the time of pouring should be diminished or increased to establish the optimum conditions for the preparation being used.

The surface of the column must be firmly packed before the sample is added. The solvent is removed to within 1 to 2 mm. of the starch. The remaining liquid is driven down at 7 cm. pressure until it clears the surface. The pressure is maintained for several minutes until the starch has fallen 1 to 2 mm. If any scum from the solvents has collected on the surface of the column, the top 1 mm. of starch should be removed with a silver spatula. The surface is tamped and leveled with a flat tipped 6 mm. glass rod. Air pressure is reapplied until the surface is fairly dry as evidenced by a frosty appearance. The surface is tamped again with the glass rod, and the process repeated until a firm, smooth surface which does not cling to the rod is obtained. For columns larger than 0.9 cm. in diameter, the preliminary packing of the surface can be performed with a spatula and the final smoothing of the surface with a glass or stainless steel plunger possessing a diameter a few mm. less than that of the chromatograph tube. Care must be taken while preparing the surface not to continue the air pressure for too long a period. If this is done, the starch will pull away from the walls of the tube and air bubbles will be seen below the surface. If the bubbles extend only a few mm., the column still gives satisfactory results. If the bubbles extend down several cm., the column should be discarded.

Except when strongly acidic solvents are being used, starch columns require treatment with 8-hydroxyquinoline to remove traces of interfering metal ions. For columns 0.9 cm. in diameter, a solution of 25 mg. of 8-hydroxyquinoline (Merck) in 2.5 cc. of the solvent used on the column is added to the tube and driven into the starch at 15 cm. pressure. When this solution has just cleared the surface, fresh solvent is added and run in until the yellowish green band of hydroxyquinoline is at least 5 cm. below the surface.

The column is then ready for the addition of the sample. If not used immediately, the columns can be allowed to drip under gravity or can be kept for at least several weeks with the solvent and separatory funnel (stop-cock closed) on the top and with the tip immersed in a test-tube filled with solvent. At the end of the experiment, the starch is conveniently removed from the narrow chromatograph tubes by a jet of water from stiff tubing (Tygon). Columns run with the solvents described in this communication should not be used more than once. The exception to this statement is the case in which the mixture applied to the column contains only the amino acids shown in Fig. 1 (or tryptophan) and is free of acids or neutral salts.

Addition of Amino Acid Sample to Column—For the curves shown in Fig. 1, an amino acid mixture[4] simulating the composition of an acid hydrolysate of β-lactoglobulin (7) was employed. A total of about 1 gm. of amino acids was dissolved in 1.5 cc. of approximately 6 N HCl and made up to a volume of 10 cc. with water. Methionine and cysteine were not included in this stock mixture, although subsequent experiments have shown that methionine may be incorporated without risk of deterioration on storage of the solution at 3°. Immediately before an experiment, the appropriate quantities of methionine and cysteine hydrochloride were made to a volume of 10 cc. with 0.1 N HCl. To a 10 cc. volumetric flask, 0.5 cc. samples of each of the aqueous solutions referred to above were added and the mixture was made to volume with *dry* butanol or 1:1 butanol-benzyl alcohol. If difficulty is encountered in dissolving the amino acid mixture upon shaking, it may be necessary to add 0.2 to 0.4 cc. of ethanol and 1 or 2 drops of 6 N HCl before the solution is diluted to volume. For a column 0.9 cm. in diameter 0.5 cc.[5] of the resulting solution, corresponding to about 2.5 mg. of total amino acids, is added to the top of the column, care being taken not to disturb the surface of the starch. The experiments illustrated in Fig. 1 were run on a column 1.9 cm. in diameter with a 2 cc. sample of the amino acid solution.

---

[4] All the amino acids employed were checked for correct elementary analysis (carbon, hydrogen, and nitrogen). Specific rotations were measured on the L-amino acids. The following preparations were used: DL-leucine, DL-isoleucine, DL-methionine, DL-valine, DL-aspartic acid, DL-threonine, glycine, L-arginine hydrochloride, L-cysteine hydrochloride, and L-cystine (all Merck); L-tyrosine and L-glutamic acid (Corn Products, recrystallized); L-proline from gelatin (8); L-alanine, L-serine, L-leucine, L-phenylalanine, L-histidine · HCl · H_2O, and L-lysine dihydrochloride prerepared from protein hydrolysates (9). We are indebted to Dr. Erwin Brand for samples of L-tryptophan and L-valine, to Dr. Karl Folkers of Merck and Company, Inc., for a sample of L-methionine, and to Dr. E. E. Howe of the same company for the DL-leucine and DL-isoleucine.

Air pressure (15 cm.) is applied and the sample driven into the column. After the liquid has reached the level of the starch, 0.2 cc. of solvent is added to rinse down the walls of the tube. The wash solution is forced into the column under pressure. The washing operation is carried out three times. Solvent is added over the column, the reservoir connected, and air pressure of 15 cm. is applied. Measurement of the effluent volumes recorded in Figs. 1, 3, and 4 was started at this time. A graduate is placed under the column and an appropriate fore fraction is usually taken before the column is placed on the fraction-collecting machine. For the first run on an unknown sample, a fore fraction is not taken and the curve is checked from the beginning for possible peaks ahead of phenylalanine and leucine.

The developmental work to establish the positions of the peaks with different solvents was done with synthetic mixtures containing one to six components. A mixture containing 50 to 150 mg. of each component was made to a volume of 25 cc. in 0.5 N HCl and 1 cc. diluted to 10 cc. with the dry organic solvent. Addition of the amino acids in different millimolar concentrations permits identification of the peaks by height and integration.

It will be noted that stock amino acid mixtures are stored in aqueous HCl solution and made to volume with the desired organic solvent immediately before use. The excess alcoholic solution is discarded. No alteration in the composition of the aqueous mixtures has been noted when they are stored at 3° for periods up to several months. A progressive decrease in the amino acid content of mixtures of amino acids stored in the acidic alcoholic solutions has been observed. Concomitantly with the decrease in free amino acid content of such solutions, the appearance has been noted of material which yields a fast moving zone on the column and gives a positive color reaction with ninhydrin. The behavior on the column of such altered amino acid solutions has been simulated by the addition, to fresh amino acid solutions, of an amino acid ester, such as phenylalanine ethyl ester hydrochloride. Some of the amino acids are esterified, therefore, upon prolonged standing in the acidic alcohol-water solvent mixtures. The brief period of contact while the solution is being added to the column is insufficient to cause losses. Nearly quantitative recoveries are obtained even when an acidic solvent is employed in the development of the chromatogram (butanol-17 per cent 0.57 N HCl, Fig.1).

---

[5] For quantitative work each pipette used was calibrated for delivery with the given organic solvent mixture.

In general, the amino acids are added to a column as their hydrochlorides in order to render the amino acids more readily soluble in the organic solvents. Of the six amino acids included in Fig. 1, all but tyrosine can be brought into solution in neutral butanol-water or butanol-benzyl alcohol-water. Chromatography of the neutral amino acid samples yields effluent curves indistinguishable from those obtained when the hydrochlorides of the amino acids are added to the column. The HCl, when included in the sample, is stripped away by the first few cm. of the starch. The fate of HCl can be visualized by incorporating a trace of methyl orange in the solvent. The HCl gives a zone which moves at about half the rate of valine and which fades out after traveling about 3 cm. as a result of gradual neutralization by the traces of basic groups present in the potato starch.

For the majority of the present experiments with protein hydrolysates, the proteins were hydrolyzed for 16 hours with 10 times their weight of boiling 6 N HCl. The most recent analyses have been carried out on hydrolysates prepared by dissolving the protein in a minimum volume of water or dilute HCl and adding 200 times the sample weight of 6 N HCl[6] twice distilled in glass. The mixture was refluxed for 16 hours on an oil bath, the oil level being kept below that of the contents of the flask. In both instances, the excess HCl was removed by repeated concentrations under reduced pressure. The hydrolysate was not filtered but was washed into a volumetric flask with small portions of water and made up to a volume corresponding to about 50 mg. of the original protein per cc. For chromatographic analysis, aliquots of this solution were diluted (0.5 cc. to 5 cc.) with the organic solvent and applied to the column in the manner outlined for the synthetic mixtures. The quantity of protein to which a given amount of hydrolysate corresponded was determined by micro-Kjeldahl nitrogen analyses on the protein and on the hydrolysate. The analyses indicated on the average about 2 per cent manipulative loss during the concentration procedure. A nitrogen analysis was also performed upon a solution obtained after 50 cc. of 6 N HCl, containing no protein, had been refluxed for 16 hours and concentrated to a volume of 5 cc. The nitrogen contributed by NH_3 in the HCl was negligible.

Collection of Effluent Fractions—In the early stages of this work, the effluent fractions were collected manually. The performance of a large number of experiments of the kind illustrated in Fig. 1 became a practicable laboratory procedure only after the construction of a fully automatic fraction-collecting machine, drawings of which are given in Fig. 2.[7,8]

---

[6] Dr. C. F. Jacobsen has discussed with us his unpublished experiments which have demonstrated the advantages of carrying out the hydrolysis in relatively dilute solution with HCl which is as free as possible from heavy metal impurities. Humin formation is reduced under these conditions.

The machine is designed to collect the effluent from the chromatogram in successive fractions of known volume. The fractions are accumulated in tubes held in the large circular test-tube rack which accommodates 80 test-tubes in each of four concentric circles. The delivery tip of the chromatograph tube makes contact with the bottom of the enlarged part of the funnel (*A*) which is mounted in the phototube housing (*B*). The funnel is a Pyrex Gooch crucible holder 32 × 160 mm. (Corning Glass Works, catalogue No. 9480), the tip of which has been constricted. Each drop falling from the tip of the funnel intercepts a light beam which is focused through a circular aperture $\frac{1}{8}$ inch in diameter upon a phototube (RCA No. 921). The tip of the funnel must be mounted above the aperture at such a height that the emerging drops intercept the light beam just as they leave the tip. If the tip is too low, hanging drops will register; if too high, the resulting free falling drop will pass through the light beam too rapidly to cause an interruption of the beam of sufficient duration to register the drop. The height of the funnel can be adjusted by sliding it in the clamp (*C*). The glass shield (*D*) minimizes evaporation from the drop.

The change in current of the phototube circuit occasioned by the falling drop is amplified through the relay (*E*) and fed into the automatic reset impulse counter (*F*). The phototube, lens, light source and relay system are available from the Langevin Corporation, 37 West 65th Street, New York (model PR-411-A), or from the Ripley Company, Inc., Deep River, Connecticut (modified 70 series, model No. 8382). The housing and mountings were constructed of bakelite. The impulse counter was purchased from the Eagle Signal Corporation, Moline, Illinois (type HZ-50A6; housing HN-84; wiring arrangment No. 1). The impulse counter can be set to record any number from 1 to 400 drops. After the preset number of drops has been registered, the counter resets itself to zero and turns on the motor (*G*) (110 volt, 60 cycle A.C., 1/20 horse power, 1725 R.P.M., 10:1 gear reduction; Boston Gear Works, North Quincy, Massachusetts, catalogue No. MB-5810-S). By means of a belt drive to the wheel (*H*), the motor turns the

---

[7] This machine was constructed with the aid of Mr. Joseph Blum of the Instrument Shop of the Institute. A photograph of the fraction collector is included in another report (10).

[8] A fraction collector based on this design is available commercially from the Technicon Company, 215 East 149th Street, New York 51. Convenient 50 tube aluminum racks with cellophane covers for handling the 18 × 150 mm. test-tubes are also available from this source.

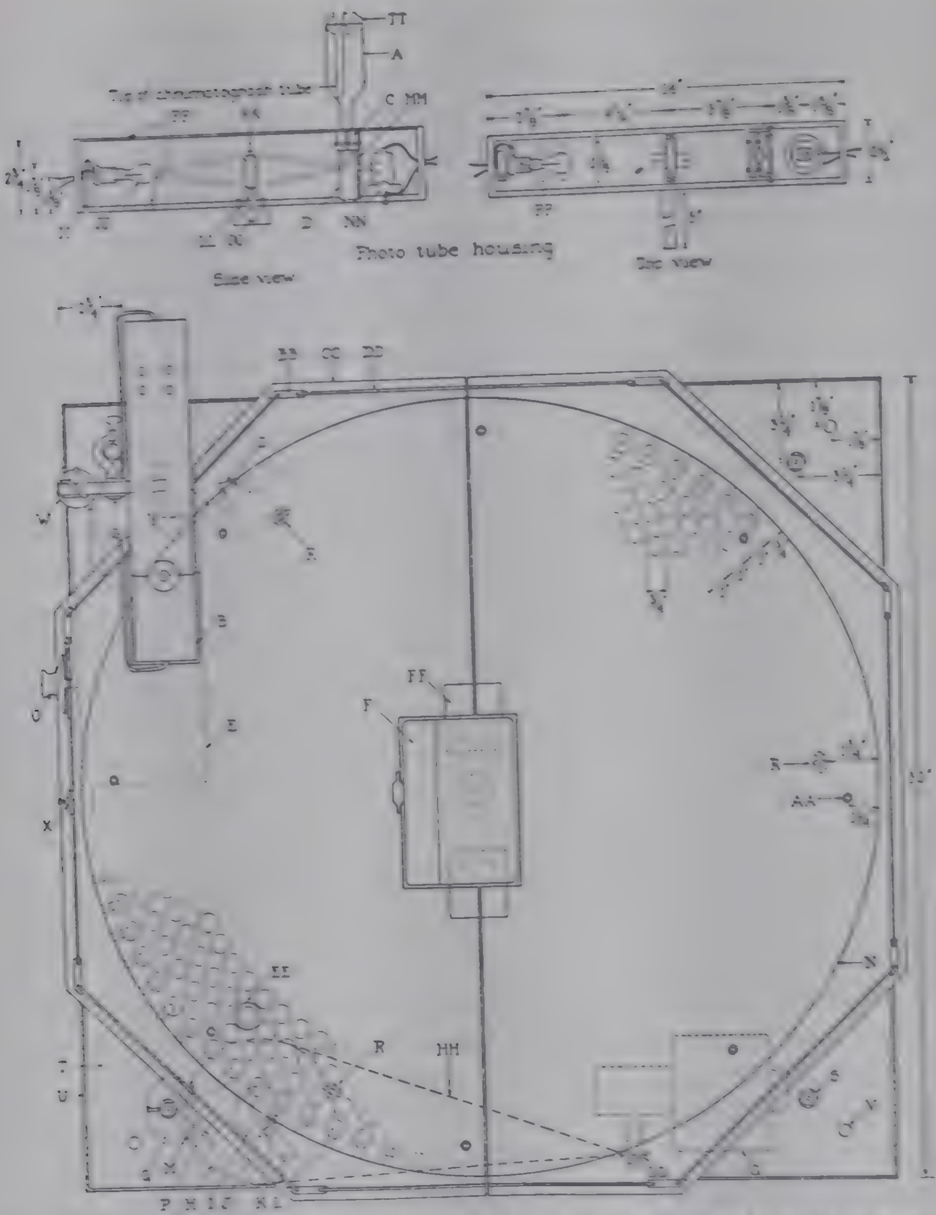


FIG. 2, *A*. Automatic fraction collector for chromatographic analysis, top view

FIGS. 2, *A* AND *B*. *A*, funnel; *B*, phototube housing (base and ends $\frac{1}{4}$ inch bakelite, sides 3/16 inch, top 3/32 inch, split into two sections at the funnel); *C*, split bakelite clamp for funnel ($\frac{3}{4} \times \frac{3}{4} \times 1\frac{1}{2}$ inches, 5/16 inch hole, thumb-screw adjustment); *D*, glass shield (1.8 cm. tubing, 2 inches long); *E*, relay held to base of machine by brackets; *F*, impulse counter; *G*, motor; *H*, 6 inch diameter pulley; *I*, brass wheel, 2 inches in diameter, 3/16 inch thick; *J*, brass engaging pin (centered $\frac{5}{8}$ inch from center of shaft, $\frac{1}{4}$ inch high, $\frac{1}{5}$ inch in diameter, with freely turning collar 19/64 inch outer diameter); *K*, slots to accommodate engaging pin (5/16 inch wide, 5/16 inch high, 1 inch deep, centers 4.5° apart, directly under the holes of the rack); *L*, arm on pulley shaft to actuate micro switch; *M*, micro switch (type 132-R, brown top); *N*, test-tube rack with four concentric circles each possessing eighty $\frac{3}{4}$ inch holes (base $\frac{3}{8}$ inch bakelite, rack 3/32 inch); *O*, sensitivity dial for relay circuit, graduated 0 to 100 over 270°, under spring

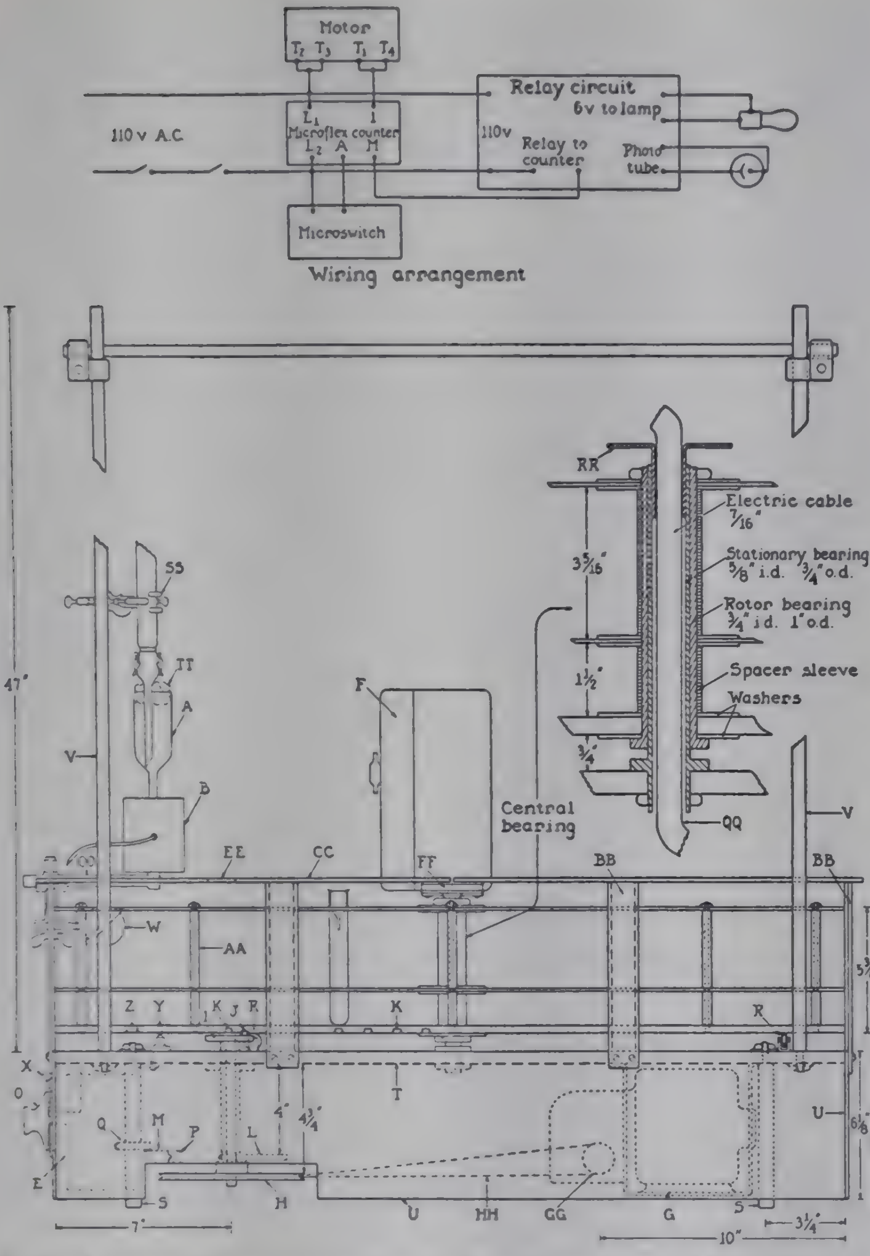

Motor
T2 T3 T1 T4
Relay circuit
6v to lamp
110 v A.C.
L1
1
Microflex counter
L2 A M
110v
Relay to counter
Photo tube
Microswitch
Wiring arrangement
RR
Electric cable 7/16"
Stationary bearing 5/8" i.d. 3/4" o.d.
Rotor bearing 3/4" i.d. 1" o.d.
Spacer sleeve
Washers
3 5/16"
1½"
¾"
QQ
V
SS
TT
A
47"
V
B
F
Central bearing
EE
CC
FF
BB
BB
W
AA
Z Y K J R
K
R
5 3/8"
X
O
Q M P
L
4"
4¾"
T
U
6 1/8"
6½"
E
S
H
U
HH
GG
G
S
7"
10"
3¼"

FIG. 2, *B*. Automatic fraction collector for chromatographic analysis, side view

tension to hold the knob in a set position; *P*, brass spring arm over micro switch; *Q*, aluminum plate to which microswitch is attached, held on leg of base by set screw; R, rollers ($\frac{3}{4}$ inch high, wheels $\frac{5}{8}$ inch diameter, $\frac{1}{4}$ inch wide); *S*, legs, $\frac{5}{8}$ inch aluminum rod turned down to 5/16 inch at the top, threaded for bolting through base; *T*, base plate, bakelite, 32 × 32 × $\frac{1}{2}$ inches; *U*, side panels, 3/32 inch bakelite (section cut out for 6 inch pulley on right side); *V*, $\frac{1}{2}$ inch Flexaframe rods, uprights turned down at the bottom to 5/16 inch, threaded for bolting through the base, with cross-braces at the top; *W*, clamp, castaloy, Fisher catalogue No. 5-764; *X*, on-off toggle switch; *Y*, toggle switch for automatic cut off; *Z*, beveled arm to throw automatic cut off switch, arm screwed to base of rack between slots; *AA*, one of eight spacers for rack, $\frac{1}{8}$ inch center rod, 5/16 inch spacing sleeves; *BB*, slotted brass supports for side panels of the cover; *CC*, wheel (*I*) which is adjacent to the rack. The upright pin (*J*) on this wheel engages one of the slots (*K*) in the base of the circular test-tube rack and moves the rack one-eightieth of a turn. A new tube is thus brought under the column. The arm (*L*) on the shaft makes contact with the micro switch (*M*) and turns off the motor after the wheels (*H* and *I*) have made one revolution. Since the counter mechanism requires that the load circuit be closed for nearly 1 second to allow time for complete resetting, it is necessary for the gear ratio to give not more than one revolution of *H* per second. The moving parts of the rack are machined from brass. The rack revolves on a sleeve bearing at the center and is supported by three symmetrically placed rollers (*R*) mounted on the base.

The fraction collector is designed for continuous duty. The counter and the relay can be replaced readily in case of failure. An extra one of each of these items should be kept on hand as replacement parts. The relay is mounted open to the air (not in a closed housing) under the base of the machine to avoid overheating. The light bulb is changed routinely after each month of continuous duty. In the installation of the photoelectric counter, it is necessary to keep the low resistance leads to the phototube short enough to bring the circuit into counting balance when most of the variable resistance (*O*) is in the circuit. The relay should close when the sensitivity dial (*O*) is turned to about 70. Accurate focusing of the light beam on the aperture is essential for this result and the aperture in the diaphragm may have to be enlarged if the light intensity striking the phototube is too low. The adjustment of the tip should be such that drops still register when the sensitivity dial is near zero. For maximum stability during operation, the dial is set about midway between the point at which the counter almost fails to register drops and the dial setting at which the counter circuit is permanently closed. The relay is designed to give an impulse of about 0.5 second duration to the counter upon interruption of the light beam for 0.01 second. The maximum counting rate with this machine is, therefore, about 2 drops per second.

⅛ inch Lucite cover, split in center, cut out around phototube housing; *DD*, 1/16 inch Lucite side panels, 7½ inches high; *EE*, 1¼ inch hole in cover, centered over third row of rack; *FF*, center supports for cover, attached to base of counter; *GG*, 1 inch diameter pulley; *HH*, 3/16 inch leather belt; *II*, split bakelite clamp for lamp socket; *JJ*, lamp, General Electric 87, 6 to 8 volts, 15 candle power, mounted with the filament vertical; *KK*, lens, 1½ inches in diameter, 2 inch focal length; *LL*, grooved lens holder of bakelite, providing a firm grip on the lens; *MM*, removable diaphragm of 3/32 inch bakelite, supporting clamp *C*, sliding into grooves in side walls of housing, with ⅛ inch diameter aperture for light beam; *NN*, phototube, RCA 921; *OO*, ½ × 5 inch supporting rod for housing; *PP*, ventilating holes in housing; *QQ*, six wire electric cable to counter; *RR*, base plate for counter, with hollow central shaft fitting tightly into stationary bearing; *SS*, clamp, castaloy, Fisher catalogue No. 5–743; *TT*, cotton packing around stem of chromatograph tube.

In the design of the fraction collector, operation on the drop-counting principle was chosen to provide rigorous control of the sample size. Experience with the machine has demonstrated that, with a dependable drop counter of the type recommended, considerable convenience and accuracy are afforded by this method of operation. For chromatographic work in general, fraction collectors operated on a time basis would probably not give as uniform fraction size, since flow rates through a column are seldom strictly constant, even when constant pressure devices are employed.

The base of the machine and the circular rack should be made of materials which do not warp and are not damaged by organic solvents or dilute acids. The rack should be of material which will not scratch photometer tubes. Bakelite has been used in the present instrument, although investigations performed recently and to be described in a forthcoming paper have revealed that this material suffers from the disadvantage that it contains ammonia or other volatile nitrogenous compounds. In chromatrographic experiments with the amino acids not included in the present work, acidic solvents, such as 2:1 *n*-propanol-0.5 N HCl, have proved extremely useful. Such solvents absorb ammonia very readily. Since as little as 0.1 γ of NH_3 per cc. is detected by the ninhydrin method, the NH_3 content of the bakelite has been troublesome. It has been possible to obtain satisfactory operation by painting the bakelite surfaces of the machine and of the phototube housing with a 2 per cent alcoholic solution of citric acid. Sheets with a dull finish take this treatment better than polished bakelite. Nevertheless, in building the fraction collector, it would be preferable to use materials which do not liberate ammonia. Provided the manufacturers cannot furnish bakelite meeting these specifications, a possibility currently being investigated, other materials might be substituted. Any material employed should first be tested by suspending a sample of it over a few cc. of 2:1 propanol-0.5 N HCl in a closed vessel for 24 hours. Analysis of neutralized 0.5 cc. aliquots of the solvent by the ninhydrin method should reveal the presence in the material of any volatile nitrogenous compounds which might be a source of interference. The Lucite cover is not resistant to butanol or benzyl alcohol and it may prove preferable to make covers from other materials. A sliding addition to the cover can also be made to fill in the space beneath the phototube housing.

In a chromatographic experiment, the starch column, to which the sample of amino acids has already been added, is mounted on the fraction collector. The solvent reservoir is attached, and the requisite air pressure applied. The stop-cock on the reservoir is clamped firmly in position to prevent it from loosening under pressure. With columns 0.9 cm. in diameter, 0.5 cc. fractions are collected. In general, in order to realize the full resolving power of the column, the size of the fractions collected should be small enough to yield at least ten points on each amino acid peak. The number of drops required for a given volume is readily determined from the weight of the liquid delivered in 10 drops from the tip, together with the density of the solvent. For small volumes, a tip which delivers 20 to 40 drops per 0.5 cc. is convenient. The size of drop delivered is not significantly changed by a 2-fold alteration in the rate of flow. The number of drops per cc. from a given tip is constant to better than 1 per cent for a given solvent over a temperature range of several degrees and is unaffected by dilute concentrations of solute in the effluent. The drop size can be increased to a maximum of about 20 drops per cc. with a fairly blunt tip of 8 mm. outer diameter. If water is being used, rather than an organic solvent mixture, a beveled tip or a constricted tip of wider bore may be required for even flow.

The machine is designed to hold 80 lipless soft glass test-tubes, 18 × 150 mm. (Arthur H. Thomas Company, catalogue No. 9446). For analytical experiments with columns 0.9 cm. in diameter, much time is saved by employing tubes that have been calibrated for use in the spectrophotometer (11). Since the whole fraction is analyzed, evaporation of some of the solvent introduces no error. If larger columns are being run for preparative purposes, uncalibrated tubes are employed and aliquots pipetted for analysis. In this case, evaporation can frequently be neglected, since with butanol it amounts to only about 50 mg. per tube in 18 hours. If desired, evaporation from the tube can be reduced almost to zero by placing a small funnel in each tube, as illustrated in the receiver in Fig. 2, *B*. The funnels can be made from 15 × 125 mm. Pyrex test-tubes with rims. In the collection of large fractions, the splashing which frequently occurs as the drops strike the funnels can be eliminated by placing a small pad of glass wool in each funnel.

The tubes are permanently numbered with a serial number and a set number and stored in sets of 100 to 200 tubes in soft aluminum racks,[8] protected from dust by covers. The filled tubes are removed from the fraction collector each day and replaced by empty receivers. The fractions are stoppered with corks until ready for analysis. A typical run for the six peaks shown in Fig. 1 requires continuous operation of the machine for about 4 days. Under these conditions the automatic cut-off arm (*Z*) is removed from the base of the rack. If it is desired to have the machine turn off after filling a prescribed number of tubes, this arm can be placed in position. As many as four columns can be run on one machine at the same time. For example, a second column can be mounted on the right side over the second or third row of receivers and the pressure adjusted to give about the same rate of flow as that of the column feeding into the photoelectric counter. The tip, with cotton packing around the stem, is protected by a glass shield fitting into an opening in the cover (*EE*, Fig. 2). For analytical experiments with columns 0.9 cm. in diameter, this arrangement can be satisfactory, since the exact fraction size does not enter into the integration of the curves. However, if the fraction size is not accurately known, the estimate of the position of a peak in terms of cc. on the abscissa of the effluent curve may be in error. If the second row experiment is a duplicate of one that has been run on the counter, the peaks can usually be identified by cross-reference to the sequence of peaks in the first determination. Columns on the inner rows can also be used for exploratory qualitative experiments.

Analysis of Effluent Fractions—When columns 0.9 cm. in diameter are employed, entire 0.5 cc. effluent fractions are analyzed by the photometric ninhydrin method outlined in the following paper (11). With larger columns, 0.1 to 0.5 cc. aliquots of the effluent fractions are pipetted for analysis. The convenience of eliminating the pipetting step points to the desirability of employing columns 0.9 cm. in diameter for experiments being run for analytical purposes.

In the plotting and integration of the curves, it is important that the base-line be correctly chosen. With each set of 50 fractions taken for analysis, six to eight fractions well ahead of or behind the peaks should be included. These fractions determine the blank reading of the effluent from the column. The positive fractions are read against the average tube of the blank series as zero.

The curves are integrated by the addition of the analytical values for the points on a given peak. In determining the mg. of sample placed on the column, calibration factors for the delivery of the pipettes with aqueous and alcoholic solutions are included (*cf.* (11), Table V).

When two or more amino acids are incompletely separated from one another, as in the case of tyrosine and valine in butanol-benzyl alcohol (Fig. 1), the amounts of each can be calculated if the overlap does not extend as far as the peak points. The calculation depends upon two experimentally observed facts; namely, (*a*) the height of a given peak is proportional to the amount of amino acid represented by the peak, and (*b*) for a given column, the height of a peak of unit area decreases approximately linearly with increasing effluent volumes. In general, the calculation is made as follows:

A_1, A_2, and A_3 = quantities in micromoles of amino acid in the first, second, and third peaks

P_1, P_2, and P_3 = corrected height of each peak in millimolar concentration from Tables I and III of the following paper (11)

V_1, V_2, and V_3 = effluent volumes at which the peaks emerge

F_1, F_2, and F_3 = color yields for amino acids A_1, A_2, and A_3

T = micromoles (leucine equivalents) obtained by integration of the combined peaks, as in Table V (11), not corrected for color yield

$$A_1 = \frac{P_1 T}{F_1\left(P_1 + \frac{V_2}{V_1}P_2 + \frac{V_3}{V_1}P_3\right)}$$

$$A_2 = \frac{\frac{V_2}{V_1}P_2 T}{F_2\left(P_1 + \frac{V_2}{V_1}P_2 + \frac{V_3}{V_1}P_3\right)}$$

$$A_3 = \frac{\frac{V_3}{V_1}P_3 T}{F_3\left(P_1 + \frac{V_2}{V_1}P_2 + \frac{V_3}{V_1}P_3\right)}$$

The recoveries of tyrosine and valine summarized in Table I have been calculated by this method by using only the terms involving P_1 and P_2 for a double peak. The calculation gives results within ±5 per cent of the theoretical recoveries in this instance. In order to apply these equations, it is necessary to have several analytical points near the peak of the curve. The recoveries are less satisfactory when the peaks are rising so rapidly that the maximum concentrations are not well defined. It is also necessary that the fraction representing the peak of each curve should contain only one amino acid. The decision as to whether the peaks are sufficiently separated to permit valid calculation can generally be made by inspection of the curves. With unknown mixtures, the applicability of the calculation should be checked by an experiment with a known mixture containing tyrosine and valine in approximately the same proportions found in the unknown. The use of partially separated peaks for analytical purposes is to be avoided whenever possible by the choice of a more favorable solvent mixture. The calculations given above have proved useful, however, for preliminary approximations, as well as in securing the data on tyrosine and valine given in Tables I, II, and III.

In some instances with larger chromatograms, it is convenient to be able to spot-test the fractions with ninhydrin-impregnated paper in order to locate the positions of major peaks and valleys. Strips of filter paper can be impregnated with a solution of 100 mg. of ninhydrin in 10 cc. of *n*-propanol and 20 cc. of 0.2 M citrate buffer, pH 5. The air-dried paper can be stored for a week or more in the dark without significant deterioration. After the application of a small drop from each fraction, the paper is warmed to 80° for development of the blue color. With butanol-benzyl alcohol, it is desirable to place a drop of water on the paper before adding the organic solvent.

Solvent Mixtures[9]—The solvent which has proved most useful for the separation of the first six amino acids (Fig. 1) contains *n*-butanol, benzyl alcohol, and water. Consden, Gordon, and Martin suggested the use of this combination of alcohols in paper chromatography (4). On starch, the mixture possesses the advantage, in comparison with butanol alone, of moving phenylalanine well ahead of leucine and isoleucine (Fig. 1). With this solvent, however, recoveries of methionine have been low, occasionally by as much as 50 per cent. Oxidation of methionine by traces of peroxides present in the benzyl alcohol appears to be responsible for this effect.[10] Quantitative recoveries of methionine are obtained when butanol is used alone. The recoveries can also be made quantitative in the butanol-benzyl alcohol solvent by the inclusion of 0.5 per cent thiodiglycol (redistilled Kromfax solvent). The solvent mixture which has been adopted for the chromatographic analysis of the first six amino acids (Fig. 1) is made up of 500 cc. of *n*-butanol, 500 cc. of benzyl alcohol, 144 cc. of water, and 5 cc. of thiodiglycol. If the thiodiglycol is not added, the water content is reduced to 142 cc.

At 25°, the above solvent is slightly undersaturated with respect to water. It is essential that all solvents meet this specification. If saturated solvents are used, slight changes in temperature may induce the separation of free droplets of water in the solvent above the chromatogram. The collection of excess water on the top of the starch column can markedly distort the amino acid peaks. The amount of water in the solvent also requires careful control in order to effect optimum separation of tyrosine and valine. If the water content of the above mixture is decreased to 138 cc., the tyrosine and valine peaks are so close together that there is no evidence of a dip or valley in the curve. The curve in Fig. 1 was obtained before this factor was fully appreciated and the result shown is therefore marginal. The increase of the ratio of benzyl alcohol to butanol to 55:45 did not permit a wider variation in the amount of water in the solvent. The relative positions of the other amino acid peaks are not affected by similar small variations in the water content. For experiments with methionine, the solvent should be used within 2 weeks after the addition of the thiodiglycol.

Butanol saturated with water contains about 170 cc. of water per liter. For the chromatogram shown in Fig. 1, *B*, an undersaturated solvent is prepared by diluting 150 cc. of water to 1 liter with *n*-butanol. The acidic solvent for Fig. 1, *C*, was prepared from 170 cc. of 0.57 N HCl made up to 1 liter with butanol. Since the presence of HCl increases the solubility of water in the alcohol, this solvent is still slightly undersaturated. The curve with the butanol-HCl mixture has been included in Fig. 1 for comparison. This solvent is unsatisfactory for many purposes, since the recoveries of amino acids may run somewhat low because of esterification.

---

[9] The organic solvents employed in this work have been of analytical reagent grade. Distillation prior to use has been found unnecessary.

[10] The peroxide content of different lots of benzyl alcohol varies as judged by the KI test. Samples giving a strongly positive reaction are not used. Even when the KI test is essentially negative, thiodiglycol must be included in the solvent.

The solvents described above are only three examples from the variety of mixtures that can be tried with starch columns, depending upon the objectives of the experiment. Further work will be reported on the use of solvents of higher water content for the fractionation of the slower moving amino acids such as alanine and glycine, and the acidic and basic amino acids. These components are still on the column at the end of the experiments described in the present communication.

DISCUSSION

Variations in Starch—The potato starch manufactured by Morningstar Nicol, Inc., which was used in the present experiments, contained 0.3 per cent ash (as sulfate), 0.05 per cent nitrogen, and 12 to 20 per cent moisture. The granules varied in size from 0.01 mm. to 0.06 mm. with the diameter of the average particle being about 0.03 mm. It has been noted that the starch is capable of neutralizing small amounts of HCl. When 20 gm. were suspended in 50 cc. of 0.01 N HCl and an aliquot of the supernatant was titrated, it was found that 1.5 cc. of 0.01 N HCl were neutralized per gm. of starch. Small quantities of ash and other impurities are extracted from the starch by the butanol and butanol-benzyl alcohol solvents. This material does not affect the analytical experiments with starch chromatograms. For isolation work, it may prove desirable to wash the starch four to six times with 6 volumes of distilled water. Before it is dried, the water-washed starch should be washed with ethanol in order to facilitate the production of a finely divided air-dried powder. For analytical experiments, the starch is used as it comes from the manufacturer, as further treatment has not served to improve its resolving power. Samples of starch washed and dried in the laboratory have actually not given quite as good column performance as the untreated commercial material.

Experiments have also been carried out with a sample of potato starch purchased from Messrs. Gordon Slater, Ltd., Manchester, England, which presumably corresponds to the material employed by Synge (6). The ash content was less than 0.1 per cent and the nitrogen content was 0.05 per cent. The particle size was significantly larger than that noted above. The average granule had a diameter of about 0.04 mm. When exposed to a saturated atmosphere of water or butanol containing 15 per cent water in a desiccator at 25°, both the Morningstar Nicol and the Gordon Slater preparations adsorbed 50 per cent of their dry weight of water or water plus a small amount of butanol. This value is higher than the value of 35 per cent reported by Synge (6).

In chromatographic work, the reproducibility of the adsorbent is a key factor in determining the usefulness of a given fractionation procedure. In the present experiments with starch, it has been found that almost identical results can be obtained with different batches of potato starch from different manufacturers when measures are taken to compensate for variations in trace metal contamination and in particle size.

The effect of small amounts of amino acid complexes of metal ions was observed in early experiments in which the first peak through the column was markedly distorted (10). It was found that this difficulty could be completely overcome by removing the interfering ions with 8-hydroxyquinoline. When a chromatogram was run with the Gordon Slater starch without treating it with hydroxyquinoline, the effect of the metal ions was not limited to the first peak, but rendered the whole chromatogram completely unsatisfactory. In butanol-benzyl alcohol the first three components emerged as one broad zone with a recovery of only 55 per cent. Prior hydroxyquinoline treatment yielded a curve almost indistinguishable from that in Fig. 1. The resolution was nearly as good and the peaks were in the same positions. With this starch, however, it was necessary to use 4 times as much hydroxyquinoline as that prescribed for the Morningstar Nicol starch. Therefore, if poor resolution is obtained with a given starch, an increase in the quantity of hydroxyquinoline may be tried. If possible, it is preferable to procure a sample of starch which requires only the minimum treatment with hydroxyquinoline. The metal effect is not correlated with the total ash of the starch. The interference is due to a minor inorganic component and it is not necessary to carry the hydroxyquinoline extraction to the point of completion. To continue the extraction to the point where no more colored complexes are eluted may require about 10 times the amount of hydroxyquinoline necessary to yield optimum amino acid resolution.

Columns packed with the Gordon Slater sample of starch flowed at about twice the optimum rate when poured originally with 30 per cent water content. The increase in flow rate correlates with the observation on the larger granule size. The satisfactory results cited above were obtained on columns poured with an initial 20 per cent water content to compensate for this difference.

Starches other than potato starch have been studied. Commercial cornstarch, after purification by acid-alcohol extraction to remove nitrogenous impurities and lipides, gave effluent curves which were qualitatively the same as those obtained with potato starch. The resolving power was much inferior and it was not determined whether improvements in the packing procedure could be made. Rice starch, with its much smaller particle size, gave flow rates that were inconveniently slow. Canna starch,[11] which posesses the largest granule size among the common starches, gave less uniform column flow when tested with a colored sample zone. On the basis of purity, availability, and functional tests, potato starch appears to be preferable to other starches for chromatographic work with amino acids.

Techniques of Column Packing—The degree of resolution of phenylalanine, leucine, and isoleucine in the butanol-benzyl alcohol solvent mixture has been used as a test in studies on the effect of variations in the packing procedure. With a poor column, the peaks are in the same positions but are lower and the spreading of the zones tends to fill in the valleys. The use of electric vibrators during packing, rotation of the column during pouring, or continuous agitation of the slurry during its introduction appeared to have little influence on the results. After the accumulation of observations on a large series of columns of varying efficiencies, the most important point in the packing procedure proved to be the water content of the starch at the time the column was prepared. Control of the water content affects the uniformity of the packing in two ways. First, the density of the starch granule decreases with increasing hydration. The density of the particles at 20 to 30 per cent water content is such that they settle very slowly and evenly in dry butanol, but the density difference is not so small that the granules tend to "float" in the solvent. The observation of a sharp settling line moving up the chromatograph tube is evidence for a satisfactory density difference. Secondly, the swelling of the starch granules from about 30 per cent water content to 50 per cent, which occurs after the column has settled to constant height and while it is equilibrating with the wet organic solvent, creates an evenly distributed internal pressure capable of reducing the intergranule interstices. The high efficiencies of these columns result in large part from the particular properties of the starch granule which make possible a packing procedure of the type employed.

As the initial water content of the starch during the pouring of the column is decreased below 20 to 30 per cent, no further significant increase in column efficiency has been obtained. The packing procedure outlined in the experimental section routinely gives columns which are essentially identical in flow characteristics and resolving power. This generalization holds for columns up to 8 cm. in diameter, the characteristics of which can be accurately predicted from runs on columns 0.9 cm. in diameter.

Rate of Flow—Decrease of the rate of flow below that prescribed in the experimental section has not yielded improved resolution. Doubling the flow rate by increasing the pressure has caused about a 10 per cent decrease in the heights of the peaks and corresponding spreading into the valleys. This increased flow rate requires the use of a piston assembly of the type described by Claesson (12) to avoid contact between the solvent and the air at the higher pressure. The solvent was added from a 100 cc. glass syringe attached to the top of the column. The syringe was enclosed in a brass container fitted to the upper part of the chromatograph tube through a rubber stopper. The maximum pressure of 15 cm., which has been adopted for the present experiments, is convenient from the standpoint of operation and gives optimum resolution.

---

[11] We are indebted to Mr. C. V. Caesar of the Stein, Hall and Company, Inc., New York, and to Dr. C. O. Beckmann for the samples of canna starch.

Variations in Amino Acid Sample—Successful fractionation of a mixture of amino acids on a starch column depends upon not overloading the column. The quantity of material that can be handled will vary with the composition of the mixture and the objective of the experiment. With the protein hydrolysates studied in the present experiments, the maximum total load of 2.5 mg. on a butanol-benzyl alcohol column causes a narrow white zone to form at the top of the translucent column. This zone is associated with the liberation of small amounts of water which cannot be absorbed by the slightly undersaturated solvent. If the amino acid load is doubled, the amount of water liberated may be sufficient to cause visible streaks to spread down the walls of the tube. Under these conditions, the peaks emerging in the effluent are broadened and resolution is inferior. The glycine, glutamic acid, lysine, ammonium chloride, and other very hydrophilic components of the mixture contribute to the limiting load in this case. The presence of significant percentages of NaCl, or other inorganic salts, may have the same effect.

The loading of phenylalanine, leucine, and isoleucine can be increased in the present experiments, if these constituents are the only components of the mixture. If the objective of the experiment is the determination of methionine, which gives a peak well separated from the rest, the load can be increased without regard to some reduction in resolving power. Also, if the objective is the isolation of components from the effluent of a large column, columns can be operated at a higher capacity and the overlapping zones discarded in working up the fractions.

Identification of Amino Acid Peaks—The effluent volume at which a given amino acid peak emerges from the column has been established in a series of experiments with simple and complex known mixtures. In experiments with relatively simple unknown mixtures, such as acid hydrolysates of purified proteins, the considerations described below make it possible to identify the familiar amino acid peaks with a relatively high degree of certainty. It should be stated at the outset, however, that in chromatographic work with unknown mixtures the only unequivocal method for the qualitative identification of the component or components in a peak is through isolation of the material in sufficient quantity to permit its characterization by independent microchemical methods. The fact that the starch column can be scaled up to 8 cm. or more in diameter indicates that this approach is feasible. Further work is required on techniques for the isolation of components from the effluent on a preparative scale.

Identification of the peaks is facilitated by the fact that the effluent volume for a given amino acid has been found to be constant to within ±5 per cent, depending upon the nature of the solvent and the weight of starch used in the preparation of the chromatogram. The position of an individual amino acid has not been influenced by the presence of other amino acid components in the mixture being fractionated. Leucine, for example, emerges at the same position when added in a synthetic mixture as when added alone. In the graphical presentation of the results, it has been convenient to measure the effluent volume from the time the sample is placed upon the column, without subtracting the initial column volume. Small variations in the positions of the effluent peaks are thereby introduced as a result of differences in the tightness of the column packing. The variations from this source are negligible in the present experiments.

Comparison of the knowns and unknowns with respect to the general sequence of the peaks and their precise positions relative to one another is of importance (Figs. 1, 3, and 4). The position of the leucine peak relative to the neighboring phenylalanine and isoleucine peaks is even more reproducible than the absolute effluent volume. The exact position of a peak on the abscissa may change slightly, for example, with small variations in the composition of the solvent. The relative positions of two peaks are seldom similarly sensitive. An exception is the case of tyrosine and valine discussed earlier.

The positions of the peaks obtained in the analysis of an unknown mixture can be checked by the addition of one or more known amino acids to the sample prior to analysis. The corresponding peaks on the effluent curve should rise without loss of symmetry, and the added amount of amino acid should be recovered quantitatively. In some cases, information on the identity of a peak can be obtained by specific color reactions. In the tyrosine range, where frequently only every second fraction is used for ninhydrin analysis, the remaining fractions containing tyrosine can be pooled, concentrated to dryness, and tested with Millon's reagent. The probability of correct identification can also be greatly increased by demonstrating that the peak from the unknown appears at the correct position when chromatographed with two or more different solvents.

When working with mixtures more complex than the usual protein hydrolysate, the problems of identification are increased. Conclusions based upon chromatographic data alone should be made with caution, particularly in the case when the mixture has not been subjected to hydrolysis. In such instances, in the absence of additional information, it cannot be assumed that a peak in the effluent curve occurring in the phenylalanine range, for example, is phenylalanine. Nevertheless, information useful as a basis for further work may be obtained. Care in the examination of each peak for its absolute position, its position relative to other peaks, and any unusual degree of asymmetry may reveal the presence of unsuspected components. If a peak should occur in a portion of the curve normally unoccupied by any of the common amino acids, it is possible to state definitely that it is not one of these substances. Similarly, the absence of an amino acid can be unequivocally demonstrated within the accuracy of the ninhydrin method. It must be borne in mind that the ninhydrin method of analysis is sensitive only to compounds containing amino groups. A single symmetrical peak on the effluent curve does not exclude the presence of ninhydrin-negative compounds with similar rates of travel on the chromatogram.

Among the possible fast moving components other than those shown in Fig. 1 is tryptophan which emerges near to phenylalanine in the three solvent mixtures. In the butanol-benzyl alcohol solvent, this amino acid is readily detected in the valley between phenylalanine and leucine, and in Fig. 4, *A* would yield a peak at an effluent volume of 38.5 cc. If the separation of phenylalanine, tryptophan, and leucine is required, experiments have indicated that they can be completely differentiated by rechromatographing the mixture on an aqueous 0.1 N HCl column (10). With acid hydrolysates of proteins, the problem seldom arises, since tryptophan is usually decomposed during the hydrolytic process. Other possible amino acid components in the leucine-valine range include dibromo- and diiodotyrosine. The latter emerges at an effluent volume of 16 cc. (Fig. 4, *A*). The presence of peptides, of course, would introduce many possible additional components.

There are no detectable differences in the rates of travel of D-, L-, and DL-amino acids on the starch column. This point has been checked with the L and DL forms of all of the amino acids covered by the present experiments, except tyrosine.

The positions of the amino acid peaks are also of significance in the theoretical interpretation of the action of the starch column. It has been pointed out that the rates of travel of the amino acids on the column do not correspond in all cases to the rates to be expected from the liquid-liquid distribution theory (10). Further studies on the acidic and basic amino acids have revealed similar discrepancies. The available data suggest that the underlying principles governing the operation of the starch column are adequately covered by the currently accepted definition of the chromatographic process. The term "liquid-liquid (partition) chromatography" does not appear to be applicable to the starch column.

Quantitative Analysis of Synthetic Mixtures of Amino Acids—The results obtained in a series of chromatograms performed on synthetic mixtures containing the eighteen amino acids most commonly found in protein hydrolysates are summarized in Table I. Recoveries on columns 1.9 cm. and 0.9 cm. in diameter have been included. The results indicate that in work with protein hydrolysates, an individual determination of a component present to the extent of 3 per cent or more of the protein is seldom in error by as much as 5 per cent. The averages of several determinations have given an accuracy of ±3 per cent.

TABLE I

Recoveries of Amino Acids from Mixtures Containing Nineteen Components

Solvent, 1:1:0.288 *n*-butanol-benzyl alcohol-water.

Mixture	Per cent recovery					
	Phenylalanine	Leucine	Isoleucine	Methionine	Tyrosine	Valine
Synthetic β-lactoglobulin hydrolysate*	101.4	101.0	103.5		97.9	102.0
	97.3	100.4	104.3		99.0	99.8
	98.6	104.5	106.8		101.8	103.0
Synthetic bovine serum albumin hydrolysate†	103.5	102.8	100.2		105.3	98.3
	103.6	101.0	102.0		106.4	96.7
	101.8	101.6	103.6		100.6	99.0
	98.4	102.0	100.0	100.0‡	101.1	100.1
	99.4	101.0	102.8	100.6‡	102.7	100.8
Average	100.5	101.7	102.9	100.3	101.8	100.0

* Corresponding in composition to an acid hydrolysate of β-lactoglobulin (Brand *et al.* (7)). Tryptophan was omitted.

† Corresponding in composition to an acid hydrolysate of bovine serum albumin (Brand (13)). Tryptophan was omitted.

‡ The solvent contained thiodiglycol. The amount of methionine present was increased to 6 times that reported for bovine serum albumin.

Composition of Hydrolysates of β-Lactoglobulin—The sample of β-lactoglobulin used in these experiments was prepared in the laboratory of the late Dr. Max Bergmann by Dr. G. Haugaard and was one of two samples recently analyzed by Brand and coworkers (7). The figure of Brand *et al.* for the nitrogen content of the ash- and moisture-free protein, 15.6 per cent, was confirmed and was employed as a basis for calculations in the present work. This protein offered opportunity for comparison of the analytical results obtained by chromatography on starch with those obtained by other methods.

The effluent curve from a sample of the hydrolysate showed no unexpected peaks (Fig. 3). The positions of the six peaks present corresponded to those of recognized components of the protein. When known amounts of phenylalanine and isoleucine were added to the hydrolysate, the peaks

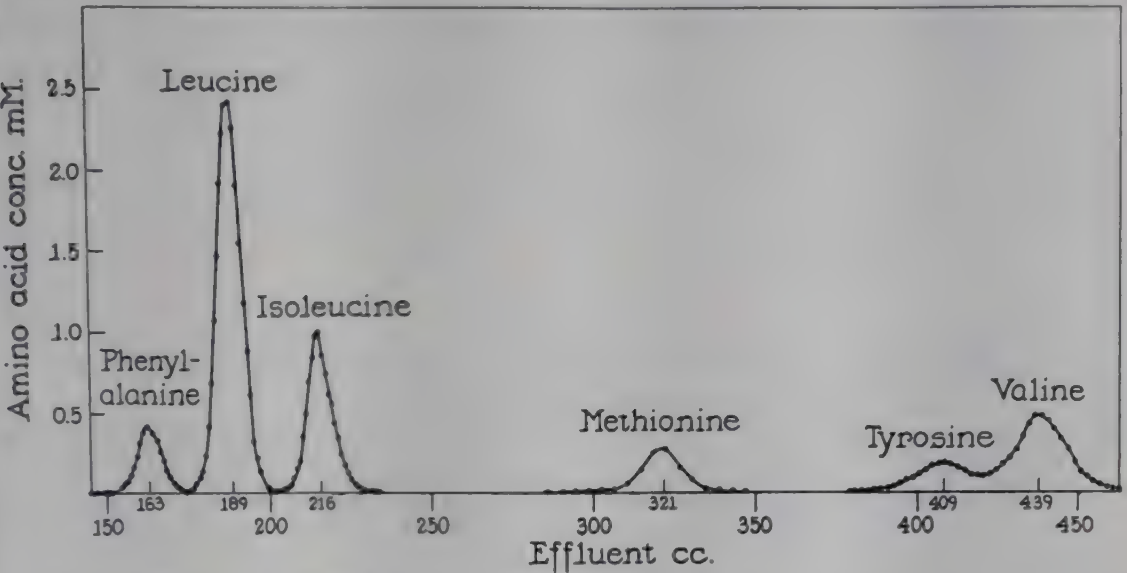


FIG. 3. Chromatographic analysis of a hydrolysate of β-lactoglobulin. Solvent, 1:1:0.288 *n*-butanol-benzyl alcohol-water (without thiodiglycol). Column, 52 gm. of starch (anhydrous); diameter, 1.9 cm.; height, 30 cm. Sample, about 20 mg. of amino acids.

TABLE II

Amino Acid Composition of Hydrolysates of β-Lactoglobulin

The numbers in parentheses refer to the bibliography.

Method of determination	Hydrolysate No.	Amino acid, gm. per 100 gm. protein				
		Phenylalanine	Leucine	Isoleucine	Tyrosine	Valine
Chromatography on starch	1	3.74	15.4	5.81	3.58	5.71
	2	3.82	15.7	6.04	3.81	5.61
	2	3.77	15.5	5.74	3.52	5.55
Average..........		3.78	15.5	5.86	3.64	5.62
Isotope dilution			15.7(14)			
Microbiological		3.54(7) 4.3(16)	15.4(15) 15.3(16)	8.4(7) 7.0(16) 8.7(17) 6.1(18)		5.8(7) 5.5(16)
Chromatography on silica gel		4.2(3)				5.8(3)
Solubility product			15.9(19)			
Photometric					3.78(7)	

assigned to these components rose accordingly to give recoveries of 99 and 98 per cent, respectively, for the added quantities. The curve was comparable to that obtained with the synthetic control (Fig. 1, *A*). The quantitative values obtained by integration of the curves are given in Table II.

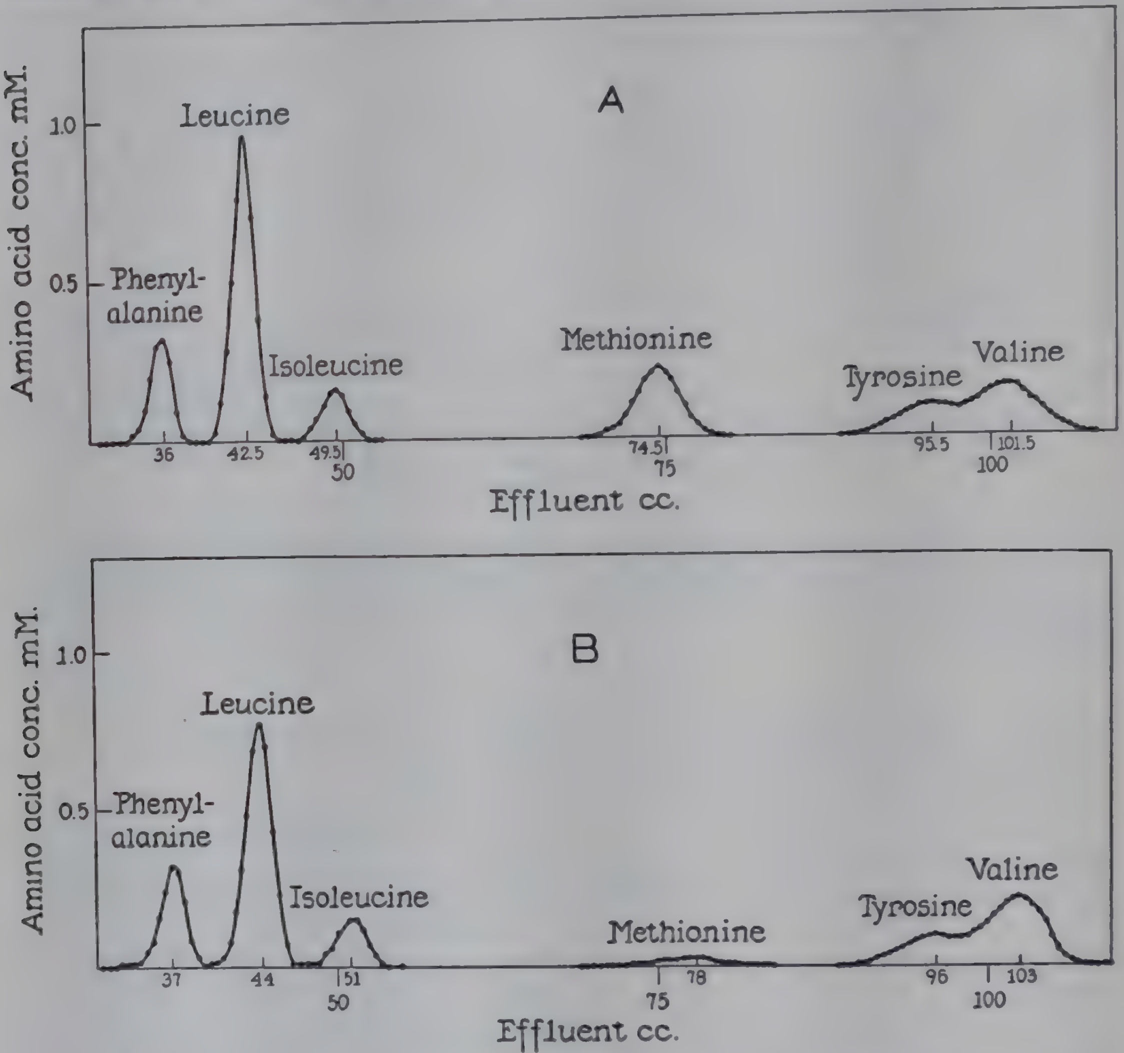


FIG. 4. Chromatographic analyses of a hydrolysate of bovine serum albumin and a synthetic mixture of similar composition. Solvent, 1:1:0.288 *n*-butanol-benzyl alcohol-water, containing 0.5 per cent thiodiglycol. *A*, synthetic mixture corresponding to an acid hydrolysate of bovine serum albumin with increased methionine content. Column, 13.4 gm. of starch (anhydrous); diameter, about 0.9 cm.; height, 31 cm. Sample, about 2.5 mg. of synthetic mixture. *B*, hydrolysate of bovine serum albumin. Column, 13.4 gm. of starch (anhydrous); diameter, about 0.9 cm.; height, 33 cm. Sample, corresponding to about 2.5 mg. of protein.

The results are in reasonable agreement with the values obtained by other methods, except in the case of isoleucine for which the chromatographic value was more than 20 per cent below the earlier microbiological values. The reason for the high results (7, 16, 17) has subsequently been ascertained and the most recent value of 6.1 per cent obtained by Smith and Greene (18) is close to the chromatographic figure of 5.86 per cent. Since the β-lactoglobulin experiments were run before thiodiglycol was incorporated into the solvent, quantitative methionine values were not obtained.

Composition of Hydrolysates of Bovine Serum Albumin—The sample of protein analyzed was obtained through the kind cooperation of Dr. Erwin Brand, and was the same preparation (Armour, lot No. 18) which was analyzed in his laboratory. The nitrogen content of the ash- and moisture-free protein, 16.07 per cent, given by Brand (13), was confirmed. An effluent curve on an acid hydrolysate of bovine serum albumin is given in Fig. 4. Integration of the peaks yielded the results given in Table III. In this case the chromatographic values for leucine, isoleucine, tyrosine, and valine are all about 10 per cent below the values given by Brand (13, 20). Phenylalanine runs about 6 per cent higher and the methionine value is essentially a check. However, the methionine peak was far too low for accurate integration. With a peak as low as that of methionine in this case, an error of 0.005 in the optical density reading for the base-line of the peak could cause an error of 15 per cent in the recovery. For amino acids present in small amounts, the accuracy can be increased by placing a larger sample on the column. For tyrosine, the value of Brand and coworkers

TABLE III

Amino Acid Composition of Hydrolysates of Bovine Serum Albumin

Method of determination	Hydrolysate No.	Amino acid, gm. per 100 gm. protein					
		Phenylalanine	Leucine	Isoleucine	Methionine	Tyrosine	Valine
Chromatography on starch	1	6.96	12.4	2.65		5.15	6.04
	1	6.42	12.4	2.67		4.76	5.86
	1	6.56	12.6	2.41		4.85	5.92
	2	6.39	11.9	2.52		4.96	5.70
	2					5.30	5.69
	3	6.56	12.0	2.74	0.92	5.18	5.95
	3	6.72	12.3	2.68	0.92	5.24	6.27
Average		6.60	12.3	2.61	0.92	5.06	5.92
Other methods		6.2*	13.7*	2.9*	0.81†	5.49‡ 5.53§	6.5*

* Microbiological assay (13, 20).

† Iodometric determination (13, 20).

‡ Photometric determination (13, 20).

§ Isotope dilution method (21).

(20) checks well the figure of Shemin (21) who employed the isotope dilution method. In the latter case, however, a different lot of serum albumin was employed. The uniformity of the discrepancy between some of the earlier results and those reported here stimulated a diligent search for systematic errors in the chromatographic procedure. Three different hydrolysates were analyzed with concordant results. The nitrogen content of the hydrolysates was determined by the same micro-Kjeldahl procedure used in the analysis of the protein. The synthetic mixtures gave excellent recoveries (Table I). There appears to be no systematic explanation for the differences.

SUMMARY

A procedure for the quantitative chromatographic separation of phenylalanine, leucine, isoleucine, methionine, tyrosine, and valine has been developed. The amino acid mixture is fractionated on a column packed with potato starch. The solvent which has been used in most of the experiments is 1:1:0.288 *n*-butanol-benzyl alcohol-water containing about 0.5 per cent thiodiglycol.

Photometric ninhydrin analyses are performed on small increments of the effluent solution to permit the construction of effluent concentration curves which reveal in detail the changes in composition of the eluate. With columns 0.9 cm. in diameter and 30 cm. in height, integration of the resulting peaks gives amino acid recoveries accurate to 3 to 5 per cent in individual determinations on 0.1 mg. quantities of a component. The average of several experiments gives recoveries to within ±3 per cent on synthetic mixtures of nineteen components corresponding in composition to protein hydrolysates. Experiments have been carried out on the determination of the amino acid composition of acid hydrolysates of samples of bovine serum albumin and β-lactoglobulin.

An automatic fraction-collecting machine is described for the collection of the large number of small effluent fractions required in this type of chromatography. The techniques for measurment of the shape and position of the emerging peaks have permitted careful comparison of different samples of starch and the factors which enter into the preparation of uniform columns. It has been possible to define procedures which have given fully reproducible resolving power from column to column and with different preparations of potato starch. The columns can be scaled up to 8 cm. in diameter without loss of efficiency.

The authors wish to acknowledge the assistance of Miss Enid Mellquist and Mr. H. R. Richter in the performance of this work.

BIBLIOGRAPHY

1. Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **35**, 1358 (1941).
2. Martin, A. J. P., and Synge, R. L. M., in Anson, M. L., and Edsall, J. T., Advances in protein chemistry, New York, **2** (1945).
3. Tristram, G. R., *Biochem. J.*, **40**, 721 (1946).
4. Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, **38**, 224 (1944).
5. Elsden, S. R., and Synge, R. L. M., *Biochem. J.*, **38**, p. ix (1944).
6. Synge, R. L. M., *Biochem. J.*, **38**, 285 (1944).
7. Brand, E., Saidel, L. J., Goldwater, W. H., Kassell, B., and Ryan, F. J., *J. Am. Chem. Soc.*, **67**, 1524 (1945).
8. Bergmann, M., *J. Biol. Chem.*, **110**, 471 (1935).
9. Stein, W. H., Moore, S., Stamm, G., Chou, C.-Y., and Bergmann, M., *J. Biol. Chem.*, **143**, 121 (1942).
10. Moore, S., and Stein, W. H., *Ann. New York Acad. Sc.*, **49**, 265 (1948).
11. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **176**, 367 (1948).
12. Claesson, S., *Ark. Kemi., Mineral. o. Geol.*, **23 A**, No. 1 (1946).
13. Brand, E., *Ann. New York Acad. Sc.*, **47**, 187 (1946).
14. Foster, G. L., *J. Biol. Chem.*, **159**, 431 (1945).
15. Ryan, F. J., and Brand, E., *J. Biol. Chem.*, **154**, 161 (1944).
16. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, **160**, 35 (1945).
17. Smith, E. L., and Greene, R. D., *J. Biol. Chem.*, **167**, 833 (1947).
18. Smith, E. L., and Greene, R. D., *J. Biol. Chem.*, **172**, 111 (1948).
19. Haugaard, G., unpublished data quoted by Stein, W. H., and Moore, S., *Ann. New York Acad. Sc.*, **47**, 95 (1946).
20. Brand, E., Kassell, B., and Saidel, L. J., *J. Clin. Invest.*, **23**, 437 (1944).
21. Shemin, D., *J. Biol. Chem.*, **159**, 439 (1945).

PHOTOMETRIC NINHYDRIN METHOD FOR USE IN THE CHROMATOGRAPHY OF AMINO ACIDS

By STANFORD MOORE and WILLIAM H. STEIN

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, June 8, 1948)

For the investigations on the chromatographic separation of amino acids outlined in the preceding communication (1), it was necessary to have available a suitable quantitative method for the determination of the concentration of amino acids in the effluent from the column. For this purpose, the method should be sufficiently general to include the determination of most of the amino acids and peptides likely to be encountered in protein hydrolysates or other material of biological origin. The method should have as high a sensitivity as possible to permit the determination of low concentrations of amino acids in the effluent from the chromatogram. Also the laboratory procedure should be fairly simple to permit the method to be applied conveniently to large numbers of effluent samples.

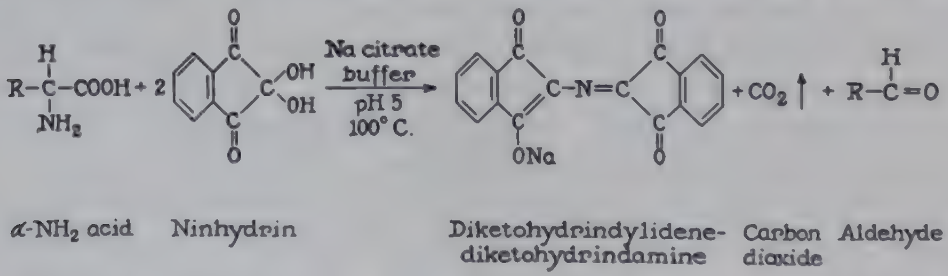
It appeared probable that a photometric method would best fulfil these requirements. The two colorimetric methods of this type which had received the most study were the procedures based on the use of β-naphthoquinonesulfonic acid and ninhydrin (triketohydrindene hydrate) as reagents. For reasons which will be described, the ninhydrin reaction was selected for further investigation.

The color reaction between α-NH_2 acids and ninhydrin has been studied extensively in the past. It has been established that colored compounds are formed not only with amino acids, but also with peptides, proteins, and other classes of substances possessing free amino groups. The reaction is known to be extremely sensitive for qualitative work. In earlier attempts to render the color reaction quantitative (2–8), however, it has been found that the color yield per microgram of amino acid decreased markedly as the concentration of amino acid was reduced. In addition, the results have not been reproducible. In the present investigations, it has been observed that, when the color development is carried out in tubes exposed to the air, these difficulties appear to result primarily from the influence of dissolved oxygen. Improved results can be obtained when the reaction is performed in tubes evacuated to 20 mm. Under these conditions, the relationship between color yield and amino acid concentration is more nearly linear, although the deviations are still marked. By the addition of a strong reducing agent directly to the reaction medium, however, the oxidative side reaction has been eliminated. In preliminary experiments the reduced form of ninhydrin, as hydrindantin (9), was added. Subsequently, it proved simpler to add stannous chloride to the reaction mixture. The stannous chloride serves to reduce part of the ninhydrin, and the preparation of hydrindantin in crystalline form is not required. Although ascorbic acid used as an antioxidant increases the sensitivity of the reaction for qualitative use (10), it is unsuitable for quantitative work because it can also give colored condensation products with amino acids (*cf.* (11)).

When conditions had been established which would give thoroughly reproducible photometric readings with a given amino acid, a study was made with several amino acids to determine the effects upon the reaction of variations in pH, temperature, time of heating, and amounts of reagents. At the same time, the mechanics of the procedure were developed to permit the analysis of large numbers of samples in a routine manner.

The method still possesses one important disadvantage. Although reproducible results are obtained for a given amino acid, the different amino acids do not all yield the same amount of color per mole. For chromatographic analysis, this is not a serious disadvantage. In those instances in which the starch column can separate an amino acid completely from the other components of the mixture, the ninhydrin reaction can be made to give quantitative values by the use of a factor appropriate for the amino acid in question.

Some of the possible causes for variations in the color yield per mole have been studied. The absorption spectra indicate that all the α-NH_2 acids (except cysteine) give the same major colored end-product. This blue coloring matter was prepared by Ruhemann (9) by the reaction of ninhydrin with alanine and by the condensation of hydrindantin with ammonia. To this compound Ruhemann assigned the structure of diketohydrindylidene-diketohydrindamine. In the present work, the product has also been isolated from the mixtures obtained in the reactions of ninhydrin with glycine and glycylleucine. The substance crystallizes as the sodium salt from the citrate buffer solution used in the present experiments. The absorption spectrum of a standard solution of diketohydrindylidene-diketohydrindamine can be compared with the spectrum of the unfractionated reaction mixtures from ninhydrin and α-NH_2 acids. The comparison shows that in the leucine reaction, for example, the colored product is formed in only 93



per cent of the theoretical yield. A modification of the method which would render the yields quantitative in all cases would be an improvement. For a given amino acid, the percentage yield of the colored product is independent of the initial amino acid concentration. This fact indicates that the low yield is characteristic of the mechanism of the reaction under the experimental conditions employed and is not due to destruction of part of the color by a trace of oxygen.

With proline and hydroxyproline, as shown by Grassmann and von Arnim (12), the reaction follows a different course than with the amino acids containing an α-NH_2 group. These two amino acids give products with a maximum absorption at 440 mμ. The present procedure can also be used to determine proline and hydroxyproline, although the sensitivity is less than in the case of the amino acids which form diketohydrindylidene-diketohydrindamine.

It has long been known that colorimetric ninhydrin methods are not specific for the NH_2 groups of amino acids. The NH_2 groups in peptides give good color development, many amines such as histamine and tyramine will react and the presence of hydrindantin, used in this procedure, causes NH_3 to give a nearly quantitative yield of the blue reaction product. For chromatographic experiments with amino acids and peptides, the generality of the reaction extends its usefulness. For work with unfractionated biological material, the lack of specificity would be a disadvantage, as was recognized by Harding and MacLean (2). The specificity of the photometric ninhydrin method is similar in a number of respects to that of the nitrous acid reaction for amino nitrogen. The method may be of value in instances when the nitrous acid reaction is useful. For the estimation of free amino acids in the presence of peptides, the photometric method, of course, lacks the specificity of the gasometric amino acid carboxyl determination of Van Slyke, Dillon, MacFadyen, and Hamilton (13).

The photometric ninhydrin method, with the present modifications which have rendered the results fully reproducible, appears to possess some advantages over β-naphthoquinonesulfonic acid procedures for those applications for which these methods are suitable. The reaction of amino acids with β-naphthoquinonesulfonic acid, as employed by Folin, recently modified by Frame, Russell, and Wilhelmi (14), and compared with the CO_2 method by Chinard and Van Slyke (15), involves the additional operation of bleaching of the excess reagent. The ninhydrin reagent solution possesses the advantage of being stable, and for routine use can be stored under nitrogen for a month or more. Fading of the color in the ninhydrin method proceeds at a much slower rate than that reported for the naphthoquinone procedure (15). The ninhydrin reaction yields the same end-product from all the α-NH_2 acids (cysteine excepted), whereas the chemistry of the β-naphthoquinonesulfonic acid reaction is less well defined and the absorption maxima of the colored products obtained from different amino acids, though similar, are not identical (14).

Apparatus

Pipettes—For the pipetting of large numbers of small samples of amino acid solutions for analysis, modified self-adjusting transfer pipettes are used in 0.05, 0.1, 0.2, and 0.5 cc. sizes.[1] The accuracy of these pipettes is increased by operating them on a manifold connected to both compressed air and vacuum lines. For long series of analyses, this arrangement is also much more convenient for the operator. The arrangement of the pipetting stand is illustrated in Fig. 1. The manifold is made from three T-tubes. The third tube is mounted behind the rubber stopper (size 13). The connections are made with soft rubber hemocytometer pipette tubing. A slight vacuum (or pressure) is applied to the micro pipette by touching the top of the appropriate T-tube and greater vacuum (or pressure) by simultaneously pressing on the adjacent rubber tubing. The stop-cocks are closed only during the adjustment of the vacuum to about 60 mm. and the pressure to about 15 mm. Before use, the tip of the micro pipette should be bent, as in Fig. 1, and fire-polished to give a delivery time of 8 to 12 seconds at 15 mm. pressure. At this rate, and with wiping of the pipette tip before delivery, the reproducibility of delivery is 0.1 to 0.2 per cent. The hold up is about 2 per cent. A series of tubes from a chromatogram is run through without washing the pipette between samples. For each solvent employed the pipette must be calibrated gravimetrically. A table of calibration factors expressed in terms of the fraction of the rated delivery is prepared for each pipette. The metal holders for the 150 × 18 mm. sample tubes and photometer tubes are cut from brass tubing of 20 mm. inner diameter.

Photometer Tubes—For the chromatographic procedure, it has been necessary to accumulate a matched set of over 1000 tubes for use with the Coleman junior spectrophotometer, model 6-A. The tubes have been selected from strain-tested soft glass test-tubes, 150 × 18 mm., without lips.[2] A solution of methyl red in 0.03 N HCl is prepared of such a strength as to give a reading of 0.60 to 0.70 on the optical density scale when read at 525 mμ against a water zero. About 100 tubes are filled with 5 to 10 cc. of the methyl red solution. It is important that all tubes receive the methyl red solution from the same reservoir bottle. Pouring the solution from tube to tube can introduce significant errors. The largest group of tubes giving readings within 0.005 unit of the same value is selected. The side of the tube facing the light source is marked temporarily at the time of the test, and subsequently with a glass-marking tool,[3] to indicate the correct position for the tube in the spectrophotometer. About a dozen of these tubes are marked as standards and reserved for use only in the checking of new sets.

---

[1] The pipettes are made to the design of Dr. P. L. Kirk by the Microchemical Specialties Company, 1834 University Avenue, Berkeley 3, California (catalogue No. 283-B).

[2] Catalogue No. 9446, Arthur H. Thomas Company, Philadelphia, Pennsylvania.

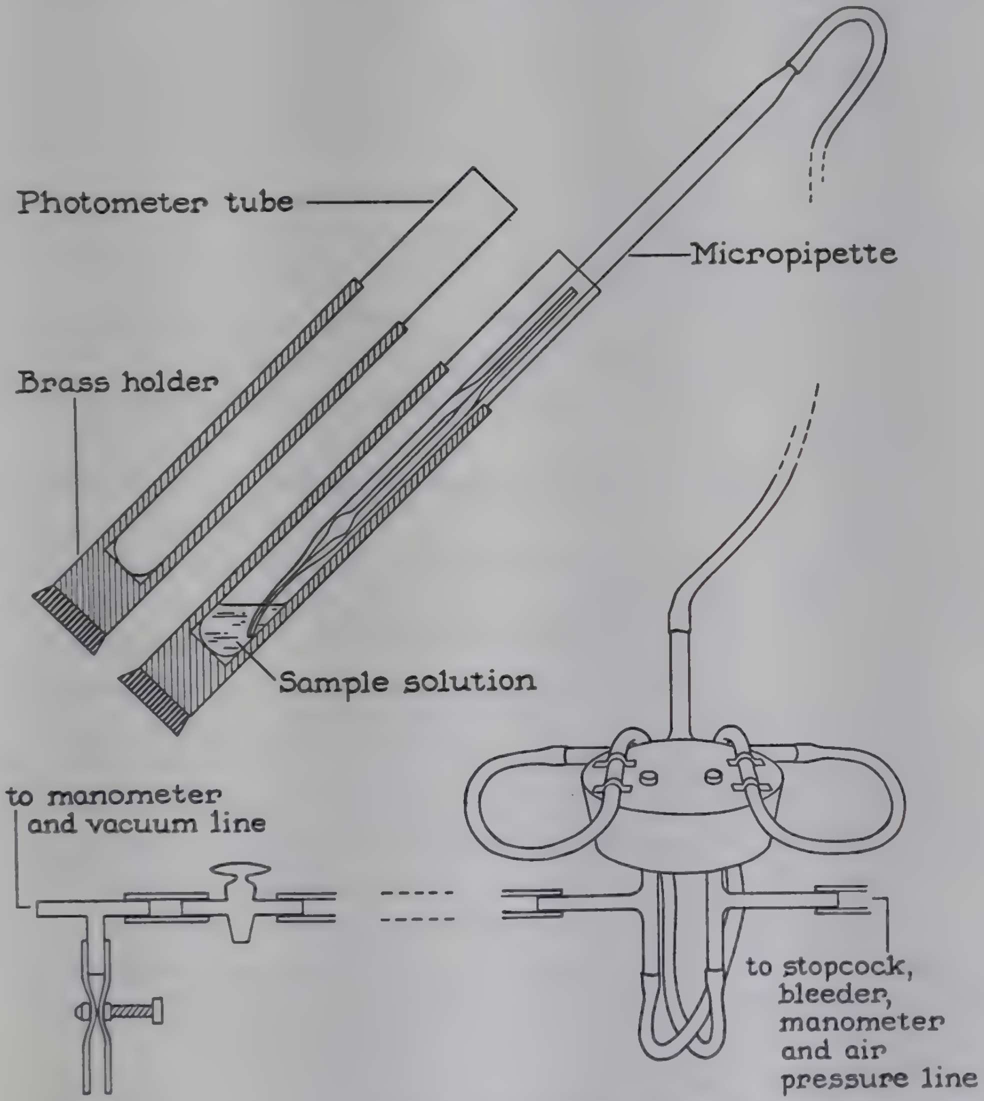

Photometer tube
Micropipette
Brass holder
Sample solution
to manometer and vacuum line
to stopcock, bleeder, manometer and air pressure line

FIG. 1. Arrangement for the pipetting of samples for analysis

In calibrating subsequent groups of 100 tubes against standard tubes filled with methyl red solution, each new tube is rotated in the instrument to determine whether it can be set to read within 0.005 unit of the standard reading. The satisfactory tubes are appropriately marked for position. The selected tubes are permanently numbered in sets of 200 tubes each. Each set can be subjected to an additional test by observing the zero reading when the tubes are filled with distilled water. In general, tubes selected by the methyl red procedure have given uniform zero readings.

---

[3] Catalogue No. 3008, Chicago Wheel and Manufacturing Company, 1101 West Monroe Street, Chicago, Illinois, with additional grinding wheels No. 5-B-44.

When measured by calipers below the rim, the tubes selected for this study had an inner diameter of 16.25 ± 0.15 mm. The outer diameter averaged 18.3 mm. Once a calibrated set has been selected with tubes of a given average diameter, such as 18.3 mm., it is necessary that subsequent lots of tubes obtained from the manufacturer have an average diameter in the same range. Under these conditions, twenty-five to 50 tubes per hundred may prove acceptable. If such specification is not made, some lots may run all high or all low.

When the sets have been handled in wooden or aluminum racks,[4] in order to protect the tubes from being scratched, and washed with boiling soap solution, the tubes have remained accurate during constant use for more than a year.

To cover the photometer tubes during the analysis, Aloe-Willett aluminum caps[5] are used.

Water Bath—A vigorously boiling water bath is required. An open bath can be used, but the amount of evaporation of the solvent (*cf.* Table III) from the tubes is less reproducible. The present experiments have been carried out in a specially designed, electrically heated, covered bath[6] with a constant level regulator. For these analyses, the bath is operated with the thermostat at the maximum setting, so that heat is constantly, not intermittently, applied. The tubes are immersed to a depth of about 2 inches. The rate of heat supply should be sufficient to bring the bath back up to 99–100° within 2 minutes after the insertion of a full rack of 50 tubes. Only one rack should be inserted at a time. The rear corners of the cover are bent downward slightly to allow the escape of steam without the collection of condensed water. A double hook handle for insertion and removal of the racks can be made from a $\frac{1}{4}$ inch metal rod.

Pipetting Machines for Reagents—For a small number of analyses, the reagents can be added by a burette or pipette. When large numbers of samples are being run, the ninhydrin solution can be stored under nitrogen in a 1 liter brown or red glass reservoir attached to a pipetting machine.[7]

[4] Suitable aluminum racks are described in the preceding communication (1).

[5] Catalogue No. JL-78300 (for 18 mm. tubes). A. S. Aloe Company, St. Louis, Missouri.

[6] Catalogue No. 405/3/R, Electric Heat Control Apparatus Company, 507 Fifth Avenue, New York 17.

[7] Model No. 40-SS-10, Brewer Automatic Pipetting Machine, Baltimore Biological Laboratory, Inc., 500 North Calvert Street, Baltimore 2, Maryland.

The flexible connections are made with $\frac{1}{8}$ inch inner diameter Neoprene tubing. A 250 cc. dropping funnel, for use in filling the reservoir, is mounted on the bottle through a 3-hole rubber stopper. The reservoir is connected to the top of a second bottle of the same size by a U-tube, and the second bottle is connected through a 2-hole stopper to a third 1 liter bottle by a U-tube which reaches to the bottom of each. The second bottle is initially filled with water, and the air in the system is replaced by nitrogen introduced through the dropping funnel at the time the apparatus is set up. The reservoir can be refilled without replenishing the nitrogen, except when the apparatus is disassembled for cleaning. The pipetting machine is equipped with a 3 cc. syringe and set for 1 cc. delivery. The valves (ungrooved) may require regrinding by hand with a fine emery or rouge to insure smooth performance with organic solvents. Fire polishing of the glass delivery tip, when small volumes such as 1 cc. are being delivered, helps to eliminate a hanging drop or back flow.

A second pipetting machine, equipped with a 10 cc. syringe, is used to deliver 5 cc. of the diluent solution from a 2 liter storage bottle. The flexible connections can be made with 3/32 inch inner diameter Tygon tubing or $\frac{1}{8}$ inch Neoprene tubing. For convenience in the analysis of large numbers of samples, the reaction mixtures are diluted with a given volume of solvent from the pipetting machine to avoid the procedure of bringing the solutions to a prescribed volume in calibrated glassware.

Reagents

Ninhydrin—To insure a low blank reading in the photometric procedure, the ninhydrin, prepared commercially according to the method of Teeters and Shriner (16), has usually been recrystallized within several months of the time of use. To 250 cc. of water, 100 gm. of ninhydrin are added. The hot solution is treated with about 5 gm. of decolorizing carbon. The filtrate is stored at 4° overnight. The ninhydrin is washed on the filter four or five times with 20 cc. portions of cold water. The air-dried crystals are stored in dark glass. The recovery is 85 to 90 per cent.

Citrate Buffer—The buffer, pH 5 (0.2 M), is prepared from 21.008 gm. of citric acid, $C_6H_8O_7 \cdot H_2O$ (reagent grade) and 200 cc. of N NaOH diluted to 500 cc. Several times this quantity can be prepared and stored in the cold with thymol. The pH of the buffer when diluted with an equal volume of water should be 5.0 ± 0.1.

Methyl Cellosolve—The samples of methyl cellosolve should give a clear solution when mixed with an equal volume of water[8] and should give a negative or very faint peroxide test with 10 per cent aqueous KI.

---

[8] Turbidity from several samples of methyl cellosolve was found to result from the fact that the solvent had been repackaged in lacquered cans. Purchase of the solvent packaged in glass or in the manufacturer's original containers is to be preferred.

Ninhydrin Solution—Dissolve 0.80 gm. of reagent $SnCl_2 \cdot 2H_2O$ in 500 cc. of the citrate buffer, pH 5. Add this solution to 20 gm. of recrystallized ninhydrin dissolved in 500 cc. of methyl cellosolve. Transfer the reagent solution to the reservoir bottle. If the system is not already filled with nitrogen, run a stream of nitrogen through the dropping funnel for about 30 minutes. When stored in this manner the solution can be kept for at least a month without deterioration. The adequacy of the preservation of hydrindantin in the reagent solution can be checked by determining the color yield on a standard amino acid solution of relatively low concentration (1 mM). If unrecrystallized ninhydrin is used, and the reagent solution is clarified with carbon, high blanks are likely to result from nitrogenous materials on the carbon.

If only a few analyses are being run at one time, and a pipetting machine is not required, the necessary small quantity of the reagent solution can be prepared for immediate use and the 1 or 2 cc. aliquots run into the photometer tubes from a burette.

Diluent Solution—Mix equal volumes of water and *n*-propanol (C.P.).

Standard Amino Acid Solutions—For the analysis of 0.1 cc. samples, 1.6 to 2.0 mM solutions of amino acids are convenient. The solutions are diluted 1:1 or 1:4 for analyses in which 0.2 or 0.5 cc. samples are required. When water-alcohol solvents are employed, the amino acids are dissolved in water and the solutions made to the appropriate volume with the dry organic solvent. A small amount of HCl is added to dissolve tyrosine and cystine. Blank solutions for each solvent mixture are prepared at the same time.

Procedure

In the case of standard solutions of amino acids, triplicate samples of the blank and the standards are pipetted into a series of photometer tubes. When the effluent from a chromatogram is being analyzed, a single sample is pipetted from each fraction. If an automatic fraction collector (1) has delivered the appropriate size of sample directly into the photometer tubes, the pipetting step is eliminated.

If the solutions for analysis are acidic, they should be neutralized (methyl red) to within ±0.1 cc. of 0.1 N NaOH. For a series of tubes from a chromatogram, a preliminary titration is made on a blank sample. Subsequently, 1.0 N NaOH is appropriately diluted so that 1 or 2 drops (0.05 to 0.1 cc.) from a burette will bring the sample within the desired limits. The prescribed number of drops of the alkali of adjusted concentration is added to each tube of the series prior to analysis. Acidic solutions will usually pick up a trace of NH_3 from the air. Since as little as 0.1 γ of NH_3 can be detected by the present procedure, it is important that the blanks and the analytical samples be handled under strictly parallel conditions.

The pipetting machine for the ninhydrin reagent is checked for delivery by running five aliquots into a 5 cc. volumetric flask. This is done daily before use of the machine, and also serves to discard the solution which has been standing in the Neoprene tubing. The delivery should be within ±0.5 per cent of the set value. One or two aliquots of the ninhydrin solution (1 cc. for 0.1 and 0.2 cc. samples; 2 cc. for 0.5 cc. samples) are run into the photometer tubes. With samples in water or in solvents miscible with water, 1 cc. of ninhydrin solution can be used for 0.5 cc. samples if accuracy to the last few per cent is not important. Aluminum caps are placed on the tubes and the tubes are shaken to mix the reagent and sample. If the shaking is done by machine, the rack can be placed for 30 seconds on a reciprocal shaker[9] operating at about 240 excursions per minute.

The rack of tubes is heated for 20 minutes in a vigorously boiling water bath. The pipetting machine for the water-propanol diluent is checked for delivery with a 25 cc. volumetric flask. 5 cc. (±0.03 cc.) of the diluent are added to each tube. The stream is directed into the center of the solution to give good mixing. The tubes are wiped and transferred to a dry rack. The tubes are shaken by hand or by machine (1 minute). Readings are taken on the spectrophotometer starting at about 15 minutes after removal from the bath. Readings are essentially constant for 1 hour after completion of the reaction. The analyses should be run in groups of not more than 50 tubes to permit the readings to be completed within this time.

The tubes are read on the Coleman junior spectrophotometer, model 6-A, at 570 mμ. The blanks are read against a reference tube of 1:1 propanol-water. The average blank tube is chosen, and the rest of the series read with the instrument set on the blank determination as zero. The blank reading should be about 0.08 to 0.10 on the optical density scale for 1 cc. of reagent and 0.15 to 0.20 for 2 cc. of reagent. Proline and hydroxyproline are read at 440 mμ.

For tubes which read near to or above 1.00, the solutions and the blank are diluted with additional 5 cc. samples of the propanol-water solution. When the volume in the tube reaches 16 to 17 cc., shaking by machine is not satisfactory, and inversion of the Neoprene-stoppered tubes is required. If the readings are still off the scale (above 1.00), samples are pipetted into other photometer tubes for further dilution.

When an analysis is completed, the tubes are rinsed with water, a monel wire screen or a perforated aluminum cover being used to permit the draining of a full rack of 50 tubes in one operation. The tubes are half filled with 0.2 per cent aqueous solution of soap flakes. The rack of tubes is heated for 20 minutes in a boiling water bath. The tubes are rinsed three times with water and dried in an oven at 110°. The boiling soap solution is required to remove the band of material that is deposited on the walls of the tubes when volatile solvents are used.

---

[9] Catalogue No. 5855, Precision Scientific Company, Chicago, Illinois.

Calculations

A standard curve is plotted for 0.1 cc. aqueous samples of leucine at six concentrations varying from 0.5 to 2.0 mm. Before being plotted, the average values are divided by the pipette calibration factor for water to give corrected readings for 0.100 cc. samples. From the graph, a table is prepared giving the millimolar concentrations corresponding to optical density readings from 0.01 to 1.00, in steps of 0.01 unit. The concentrations are multiplied by 11.1/6.1 and 16.1/6.1 to give concentrations corresponding to the readings obtained after dilution of the 6.1 cc. with one or two additional 5 cc. aliquots of the diluent. A condensed format of the standard table obtained with tubes possessing an inner diameter of 16.25 mm. is given in Table I, which covers concentrations up to 5.4 mm. The procedure can be extended by manual dilutions to much higher concentrations. The curve follows Beer's law through readings up to an optical density of about 0.50. There is a deviation of 4 per cent from the straight line relationship at an optical density of 1.0.

TABLE I

Relationship of Optical Density to Leucine Concentration (Condensed Table of Leucine Equivalents)

Determined on 0.100 cc. aqueous samples in photometer tubes of 16.25 mm. inner diameter.

Spectrophotometer reading, optical density × 100	Leucine concentration, mm per liter			Spectrophotometer reading, optical density × 100	Leucine concentration, mm per liter		
	Volume of diluent added				Volume of diluent added		
	5 cc.	10 cc.	15 cc.		5 cc.	10 cc.	15 cc.
10	0.196	0.357	0.518	60	1.18	2.15	3.11
20	0.392	0.714	1.03	70	1.39	2.53	3.67
30	0.588	1.07	1.55	80	1.61	2.93	4.25
40	0.784	1.43	2.07	90	1.83	3.33	4.83
50	0.980	1.78	2.59	100	2.05	3.73	5.42

For the other amino acids and related compounds, the color yields per mole can be expressed relative to the leucine value as 1.00. For 0.1 cc. aqueous samples, the millimolar concentrations read from Table I, corrected for pipette delivery, were divided by the millimolar concentrations of the standard solutions to give the yields summarized in Table II. Each amino acid and peptide listed in Table II was checked for correct elementary analysis and, whenever possible, for optical rotation (*cf.* (1)). The other substances were obtained from commercial sources and were not purified before analysis.

For other than 0.1 cc. aqueous samples, the values given in Table I for millimolar concentration corresponding to a given spectrophotometer reading require correction for the changes in volume involved. Before calculating the correction factors to be applied to the analysis of samples containing volatile organic solvents, it is necessary to determine gravimetrically the amount of evaporation during the analysis by weighing tubes before and after heating the reaction mixture for 20 minutes under the experimental conditions employed in an actual determination. With butanol-water and propanol-water samples, essentially the entire 0.1 to 0.5 cc. sample of solvent evaporates during the heating process. If acidic

TABLE II

Color Yields from Amino Acids and Other Compounds on Molar Basis Relative to Leucine

Determined on 0.1 cc. aqueous samples of 2.0 mM solutions; heating time, 20 minutes; read at 570 mμ.

Compound	Color yield	Compound	Color yield
Alanine	1.01	Glutathione	0.76
Arginine	1.00	Glycine ethyl ester	1.00
Aspartic acid	0.88	Glycyltyrosine	0.88
Citrulline	1.03	Glycylphenylalanine	1.04
Glutamic acid	1.05	Glycylglycine	0.89
Glycine	1.01	Glycylleucine	1.05
Histidine	1.04	Leucylglycine	0.92
Isoleucine	1.00	Phenylalanylglycine	0.97
Leucine	1.00	Phenylalanine ethyl ester	0.98
Lysine	1.12	Histamine	0.65
Methionine	1.00	Taurine	0.97
Phenylalanine	0.88	Tyramine	0.64
Serine	0.94	Sarcosine	0.84 *Ca.*
Threonine	0.92	Glucosamine	1.00
Tyrosine	0.88	Creatine	0.03
Valine	1.02	Creatinine	0.03
Cysteine	0.15 *Ca.*	Dibenzylamine	0.04
Half-cystine	0.54	Glycine anhydride	0.01
Tryptophan	0.72 *Ca.*	Urea	0.03
Proline	0.05	Adenine	0.00
Hydroxyproline	0.03	*p*-Aminobenzoic acid	0.00
Ammonia	0.98 *Ca.*	Diethylbarbituric acid	0.00
Asparagine	0.94	Glucose	0.00
Glutamine	0.99	Uric acid	0.00

samples are neutralized before analysis, the volume of NaOH solution added must be included in the calculation. When each term is expressed in cc., the correction factors (F) are calculated as follows:

$$F = \frac{\begin{array}{c}\text{(Sample volume + neutralizing solution}\\ \text{+ ninhydrin solution + diluent)} - \text{(loss by evaporation)}\end{array}}{1.1 + \text{diluent}} \times \frac{0.100}{\text{sample volume}}$$

Representative factors for two organic solvents are given in Table III. As a first approximation, these factors, used in conjunction with the relative yields of color listed in Table II, give satisfactory results for most of the amino acids in these solvents, if an accuracy greater than ±5 per cent is not required. The color yields given in Table II and the factors listed in Table III may also be used for approximate results with other solvents that may be tried with starch chromatograms. Under such circumstances, the factors given in Table III for water or the butanol-benzyl alcohol solvent may be used for relatively non-volatile solvents, and the butanol factors for volatile solvents.

TABLE III

Factors for Different Sample Sizes and Solvent Systems

Factors by which the millimolar concentrations from Table I are to be multiplied to give corrected leucine equivalents.

Solvent	Sample size	Ninhydrin solution	Loss by evaporation		Factor†		
					Volume of diluent added		
					5 cc.	10 cc.	15 cc.
	cc.	cc.	mg.	cc.*			
Water	0.1	1	(14)‡		1.000	1.000	1.000
	0.2	1	(19)		0.508	0.504	0.502
	0.5	2	(19)		0.246	0.225	0.217
Butanol-water§	0.1	1	94	0.10	0.984	0.991	0.993
	0.2	1	194	0.20	0.492	0.495	0.496
	0.5	2	395	0.45	0.231	0.217	0.212
Butanol-benzyl alcohol-water ‖	0.1	1	41	0.03	0.996	0.998	0.998
	0.2	1	62	0.05	0.504	0.503	0.502
	0.5	2	79	0.07	0.244	0.224	0.216

* Approximate.

† To be divided by the calibration factor of the pipette.

‡ The small loss from water samples is subtracted from the loss with organic solvent samples in the calculation of approximate volume change.

§ Butanol-water containing 150 cc. of water per liter ($d^{25} = 0.838$).

‖ Butanol-benzyl alcohol-water 1:1:0.288 by volume ($d^{25} = 0.936$).

However, for accurate quantitative work with those solvents which are selected as optimum for chromatographic analyses, the exact degree of evaporation must be determined experimentally, and the color yields obtained from a given amino acid must be checked by the user with standard solutions made up in the organic solvent. As may be seen from Table IV, the yields thus obtained may differ by a few per cent from the values obtained with aqueous samples given in Table II. The yields should be determined on the same size of sample being used in the chromatographic experiments, although no differences have been observed between 0.1 cc. and 0.5 cc. samples in the solvents studied thus far. For accurate work with solvents other than water, therefore, the concentration of amino acid given in Table I is multiplied by the appropriate factor from Table III and divided by the appropriate color yield from Table IV.

TABLE IV

Color Yields from Amino Acids in Organic Solvent Solutions on Molar Basis Relative to Leucine in Water

Determined on 0.1 cc. to 0.5 cc. samples; heating time, 20 minutes.

Amino acid	Color yield	
	Butanol-water solvent	Butanol-benzyl alcohol-water solvent
Leucine	0.99	1.01
Isoleucine	1.00	1.03
Phenylalanine	0.85	0.86
Tyrosine	0.86	0.87
Methionine	1.00	1.01
Valine	1.01	1.03

The factors given in Table III and the color yields listed in Table IV are fully reproducible when once determined under given experimental conditions. Except with NH_3 and tryptophan, it is not necessary to run controls with each batch of analyses. If this were not the case, the application to chromatography would be rendered unduly complicated by a need for repeated controls. An occasional check on the recovery of leucine from a known solution serves to confirm the reproducibility of the procedure.

The calculation of the recovery of leucine from a synthetic mixture which has been subjected to chromatographic analysis is given in Table V. Suitable data sheets are mimeographed to facilitate the handling of the results. In this example, the automatic fraction collector has been used with a column 0.9 cm. in diameter to deliver approximately 0.5 cc. samples directly to photometer tubes.

When aliquots are pipetted for analysis from larger effluent fractions, the summations of the uncorrected millimolar concentrations are multi-

TABLE V

Data Sheet, Determination of Leucine

Solvent, 1:1:0.288 butanol-benzyl alcohol-water; ninhydrin solution, 2 cc.; fraction collector, 25 drops = 0.504 cc.; entire fraction analyzed; wave-length, 570 mμ; readings recorded as optical density × 100.

Fraction No.	Volume of diluent, 5 cc.		Volume of diluent, 10 cc.	Volume of diluent, 15 cc.	Uncorrected amino acid concentration[†]
	Read against 1:1 water-propanol	Read against blank fraction[*]			
					mM
11	22.0	0	0	0	
13	22.2	0			
15	21.1	−1			
35	22.0	0			
36	22.0	0			0.00
37	23.5	1.5			0.03
38	29.0	7.0			0.14
39		24			0.47
40		57			1.12
41		100	62		2.22
42		140	90		3.33
43		150	102	81	4.30
44		130	84		3.08
45		77			1.54
46		28			0.55
47	28.5	6.5			0.13
48	22.0	0			0.00
49	22.5	0.5			
50	22.0	0			

Integration: Fraction 43. 4.30 × 0.216 × 0.5[‡] = 0.464
Sum of Fractions 41, 42, and 44. 8.63 × 0.224 × 0.5 = 0.967
" " " 37-42, and 46-47. 3.98 × 0.244 × 0.5 = 0.486
Total = 1.917 micromoles.

Correction for color yield from leucine in this solvent, 1.917/1.01 = 1.898 micromoles = 0.249 mg. of leucine.

Leucine added, standard aqueous solution of a mixture of amino acids 40 mM with respect to leucine; 0.495 cc. (0.5 cc. pipette, delivery 99 per cent) diluted to 5.00 cc. and 0.485 cc. (0.5 cc. pipette, delivery 97 per cent) placed on the column in butanol-benzyl alcohol. Theoretical yield = 1.920 micromoles = 0.252 mg.; recovery = 98.8 per cent.

---

[*] Fraction 11.

[†] From the expanded form of Table I.

[‡] If 0.5 cc. samples are pipetted, the factor becomes 0.216 × (volume of effluent fraction)/(pipette calibration factor).

plied by the appropriate factors from Table III and by the exact volume of an effluent fraction to give micromoles of amino acid. When the whole fraction is analyzed, as in this example, the sample volume is equal to the volume of an effluent fraction, and the two terms cancel out. At large effluent volumes, when the peaks cover twenty or more fractions, integrations can be obtained from the analysis of every second fraction.

In order to obtain a graphic picture of the fractionation, the uncorrected millimolar concentrations are used directly for preliminary plotting of effluent concentration curves. The graph gives information on the symmetry of the curves and the degree of fractionation. The curves are plotted before decision is made on the division of the peaks for integration. For publication, the curves in the preceding paper (1) have been replotted, each point being corrected to "leucine equivalents" by means of Tables I and III. When the peaks are completely separated, the curves could also be corrected for color yield, but in the case of incomplete separation of the components, this is not possible. In the preceding paper (1) the method is given for the calculation of the amounts of each component in a series of partially overlapping peaks.

Accuracy—When the calibrations have been carefully made both for the pipettes and the photometer tubes, the readings on 0.2 micromole of an α-NH_2 acid can invariably be reproduced to within 0.02 optical density unit, corresponding to an accuracy of about 2 per cent. In the chromatographic analyses, it is necessary to work, in part, below this optimum concentration range. In a large series of chromatograms, integration of the effluent curves has given recoveries of 100 ± 3 per cent, under favorable conditions, and 100 ± 5 per cent for peaks markedly below the optimum average concentration (1).

Reaction with Proline and Hydroxyproline—The preceding method can be used for the determination of proline and hydroxyproline by measuring the yellowish red products of the reaction at their absorption maximum of 440 mμ. However, the optical density readings are only one-fourth and one-seventh, respectively, of those obtained with equimolar solutions of the α-NH_2 acids. The color development is only 80 to 90 per cent complete in 20 minutes at 100°. Standard curves can be prepared for proline and hydroxyproline with a 30 or 40 minute heating period. In the chromatographic analysis, the tubes are usually heated for only 20 minutes along with the rest of the effluent samples. As a first approximation, millimolar concentrations can be calculated from Tables I and III, just as in the case of readings at 570 mμ, and the values can be converted to proline by multiplying by the factor 3.7 and to hydroxyproline by multiplying by the factor 7.2. The measurement of proline by the ninhydrin reaction is of course possible only when this amino acid is completely separated from the other amino acids, as it frequently is on the starch chromatograms. On the other hand, in view of their low absorption at 570 mμ, small amounts of proline and hydroxyproline can be present simultaneously with α-NH_2 acids without giving significant interference.

Reaction with NH_3—In the oxidative deamination of amino acids by ninhydrin, 1 equivalent of the reagent is reduced in the course of the formation of diketohydrindylidene-diketohydrindamine (9). If oxygen could be completely eliminated by evacuation of the system, the maximum color yields would be obtained from amino acids without the addition of any further source of reduced ninhydrin. The yield from NH_3 under these conditions would be low, since, of itself, NH_3 does not give rise to the reduced ninhydrin which is essential for the formation of the colored complex. This accounts for the fact that NH_3 does not react positively in a number of the colorimetric ninhydrin procedures that have been used (4, 5, 7). When hydrindantin exists preformed in the reaction mixture, however, as it does in the present procedure, the color yield from NH_3 is in the same range as that from the amino acids. In contrast to the amino acid reaction, which is independent of the concentration of hydrindantin above a certain minimum level, the color yield from NH_3 increases with the hydrindantin concentration. With the reagent solution used in the present procedure, the color yield from NH_3 reaches about 90 per cent of its maximum value. Since different batches of reagent solution may vary somewhat in hydrindantin content, a control determination on a known NH_4Cl solution must be run simultaneously if it is desired to obtain accurate values on NH_3 solutions by the photometric ninhydrin method.

Experiments on Color Development

Isolation of Diketohydrindylidene-Diketohydrindamine—The product of the reaction of ninhydrin with glycine at pH 5 was prepared in order to compare its absorption spectrum with that of the unfractionated reaction mixture obtained in the ninhydrin analysis.

The amino acid (75 mg.), dissolved in 10 cc. of water, was heated for 20 minutes at 100° with 700 mg. of ninhydrin dissolved in 20 cc. of citrate buffer, pH 5 (0.2 M). The product (245 mg.) which crystallized from the cooled solution corresponded to 75 per cent of the theoretical yield of the sodium salt of diketohydrindylidene-diketohydrindamine. The same procedure carried out with the peptide leucylglycine (188 mg.) gave the same product in 40 per cent yield. For analysis the sodium salt (50 mg.) was recrystallized from about 15 cc. of hot 1:1 water-*n*-propanol.

$C_{18}H_8O_4NNa$ (325.2).	Calculated.	C 66.5, H 2.5, N 4.3, Na 7.08
Prepared from glycine.	Found.	" 66.2, " 2.6, " 4.4, " 7.07
" " leucylglycine.	"	" 66.6, " 2.6, " 4.3, " 6.94

Absorption Spectra—The absorption curves have been determined with 0.1 cc. samples of 2 mM aqueous amino acid solutions. The final volume of the reaction mixture was 6.10 cc. in photometer tubes of 16.25 mm. inner diameter. In Fig. 2 the curves for leucine, serine, and ammonia are compared with the absorption spectrum obtained from an equimolar solution of the crystalline sodium salt of diketohydrindylidene-diketohydrindamine. The solution was prepared by dissolving 1.065 mg. of the sodium salt in 100 cc. of a mixture of the ninhydrin solution and the propanol-water diluent in the proportions of 1:10 and was read against this solvent as the blank. The absorption spectra support the conclusion that the α-NH_2 acids and ammonia yield diketohydrindylidene-diketohydrindamine under the conditions of the determination. The curves obtained with the other α-NH_2 acids, except cysteine, and with peptides, are similar to those shown in Fig. 2. The major end-product is the same in all cases, exhibiting an absorption maximum at 570 mμ. The differences in the color intensities obtained with the individual amino acids arise from variations in the yield of this product. Relative to leucine, the reading of the pure sodium salt corresponds to a color yield of 1.07 (Table II). On this basis, leucine yields about 93 per cent of the theoretical amount of this product. The yields for phenylalanine and glutamic acid, for example, are 82 and 98 per cent.

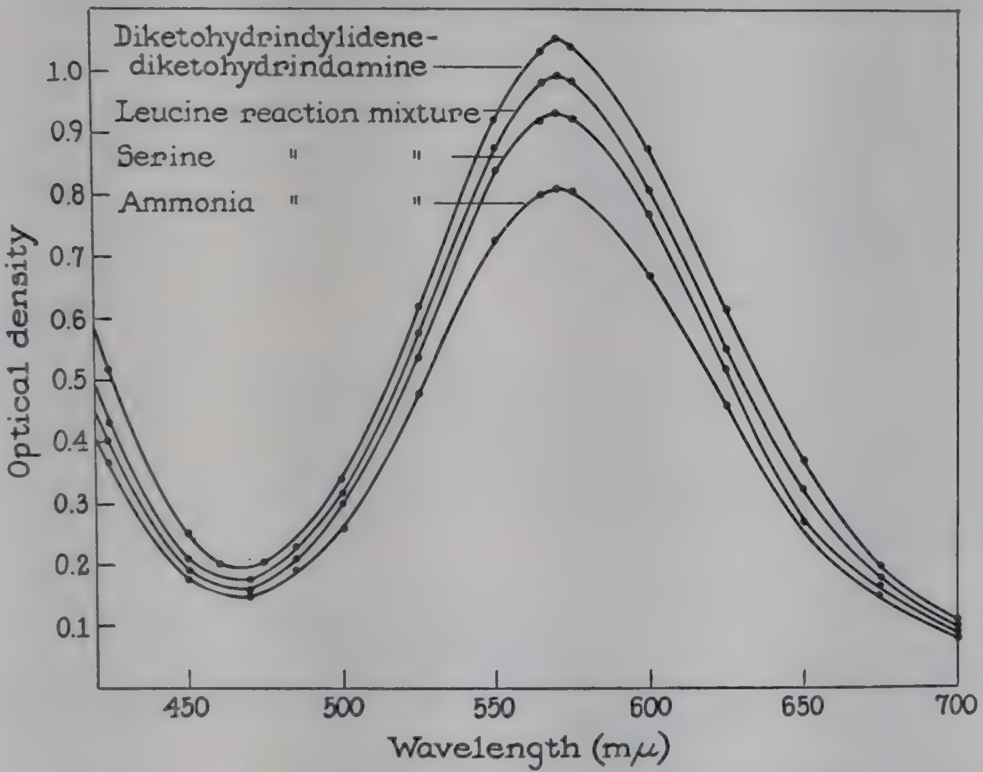


FIG. 2. Comparison of the absorption spectrum of diketohydrindylidene-diketohydrindamine with the spectra obtained after the reaction of ninhydrin with equimolar amounts of leucine, serine, and ammonia.

Color development with ninhydrin is not specific for amino acids, since a variety of primary amines and some secondary amines will give significant amounts of color. The structure of the end-products in these cases remains to be determined. The absorption curves obtained with histamine and with the N-methyl-substituted amino acid, sarcosine, are given in Fig. 3. Both compounds yield products with absorption maxima at 570 mμ.

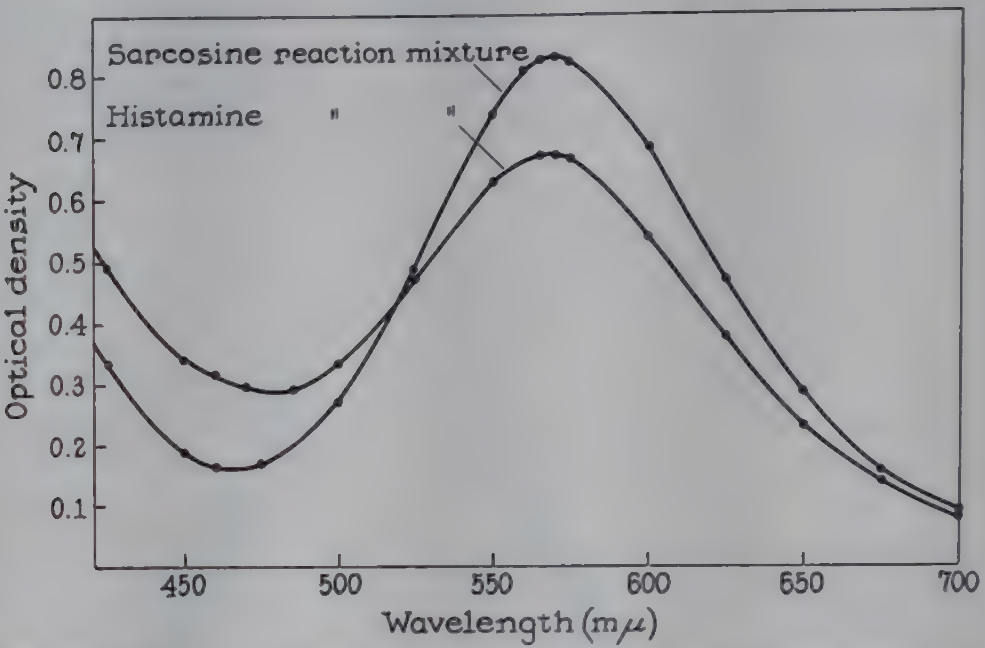


FIG. 3. Absorption spectra obtained after the reaction of ninhydrin with sarcosine and histamine.

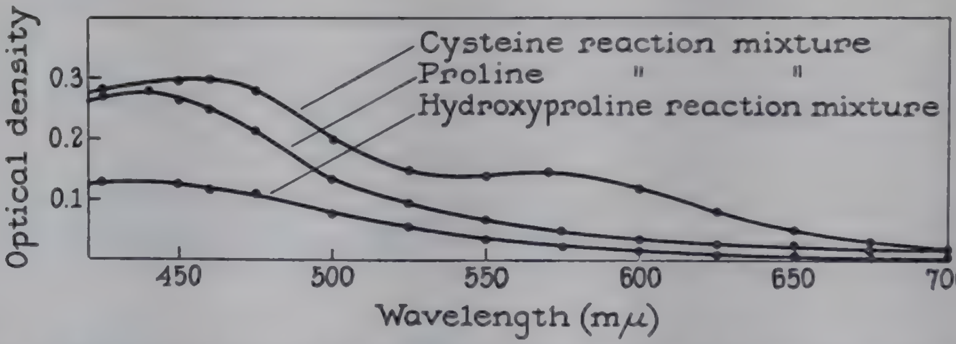


FIG. 4. Absorption spectra obtained after the reaction of ninhydrin with proline, hydroxyproline, and cysteine.

Cysteine, which has been mentioned as an exception to the general reaction of the α-NH_2 acids, gives an absorption curve which is somewhat similar to that obtained with proline and hydroxyproline (Fig. 4). Neutral cysteine solutions, after standing for 24 to 48 hours, give the same color yield as cystine, with maximum absorption at 570 mμ. It is possible that the small amount of absorption in the range of 570 mμ observed with fresh solutions of cysteine may be attributed to the presence of some cystine in the reaction mixture. The compounds yielded by the prolines under these conditions presumably correspond to the structures assigned by Grassmann and von Arnim (12).

Variation of pH—The variation of color yield with the pH of the aqueous citrate buffer is shown in Fig. 5. The absorption maximum for the α-NH_2 acids is at 570 mμ over the pH range studied. The maximum color yield from leucine is obtained at pH 5, which has been chosen for the general procedure.

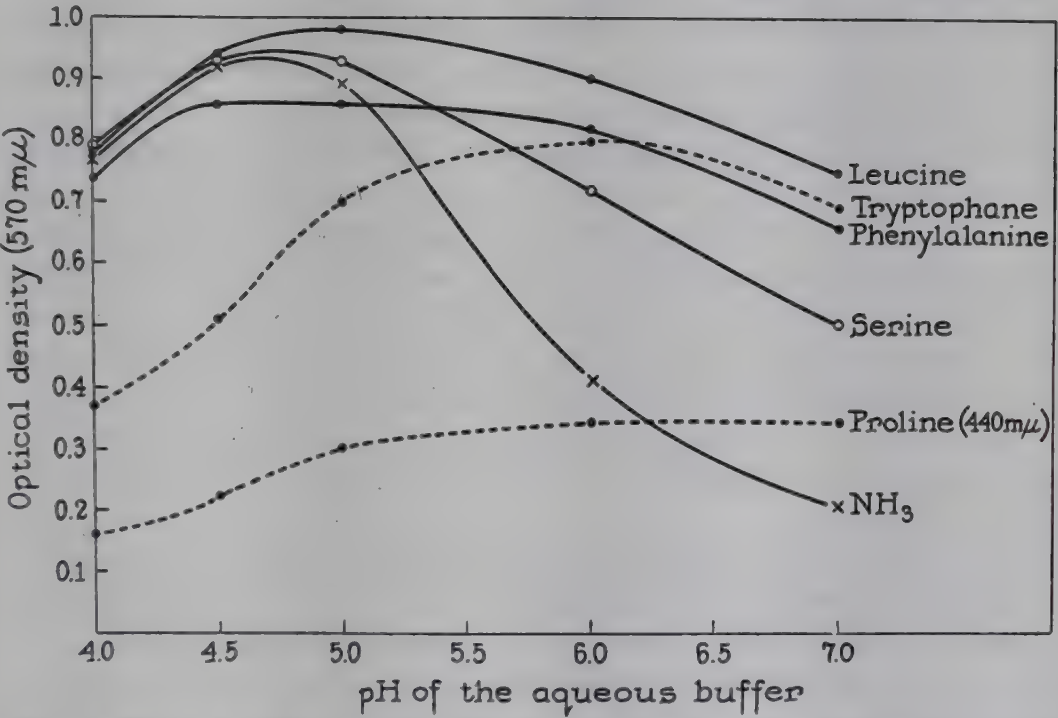


FIG. 5. Effect of pH on the intensity of color obtained after the reaction of ninhydrin with amino acids and ammonia.

The pH optima for the other α-NH_2 acids fall close to this value, with the exception of tryptophan which gives a maximum yield at pH 6. For most of the amino acids, a change in pH of 0.1 unit at pH 5.0 introduces less than a 1 per cent deviation in the optical density reading.

Rate of Reaction—The rates of color development have been determined for representative α-NH_2 acids over a range of pH from 4 to 7. In all cases, at 100° the reaction was complete in less than 20 minutes. The color yields were unaltered by an increase in the ninhydrin concentration. This result parallels the observations on the heating periods required for the complete liberation of CO_2 from amino acids at a ninhydrin concentration of 20 mg. per cc. in the gasometric method of Van Slyke, Dillon, Mac-

Fadyen, and Hamilton (13). A more detailed study of the rate of color development has been made at pH 5. Constant readings are obtained with leucine in 5 minutes and with alanine in 10 minutes. The reaction with glycylphenylalanine and glycylleucine is complete in 20 minutes. On the other hand, phenylalanylglycine and leucylglycine reach only 90 per cent of completion in this time. Primary amines, such as ethylamine and ethanolamine, react still more slowly. The time of 20 minutes has been chosen as a heating period which gives constant readings with all of the α-NH_2 acids and can be expected to give reasonably high readings with most peptides possessing a free NH_2 group.

Temperature—The color yields are lower if the reaction is carried out at temperatures below 100°. For leucine, isoleucine, tyrosine, and phenylalanine, the optical densities were 4 per cent lower when the analysis was carried to completion in a water bath maintained at 95°.

Stability of Color—The rate of fading of the blue color is illustrated by the following average readings obtained on leucine samples at the specified times after removal of the photometer tubes from the heating bath: 15 minutes 0.835, 30 minutes 0.830, 45 minutes 0.835, 60 minutes 0.835, $1\frac{1}{2}$ hours 0.815, $2\frac{1}{2}$ hours 0.810, 4 hours 0.795, $5\frac{1}{2}$ hours 0.785, and 22 hours 0.710. Thus, the color is stable for about 60 minutes, after which time there is a gradual fading, averaging approximately 1 per cent per hour. The end-product is not highly sensitive to oxidation by air, whereas, as noted below, an intermediate in the reaction appears to possess much greater sensitivity.

In early experiments, water was used as the diluent and marked fading was noted as a result of precipitation of the sodium salt of diketohydrindylidene-diketohydrindamine. The use of 1:1 water-*n*-propanol as the diluent serves to keep the relatively insoluble reaction product in solution.

Effect of Stannous Chloride—When 2 mM leucine samples are analyzed with a ninhydrin solution from which the stannous chloride has been omitted, the color yield is about half that obtained in its presence. At lower leucine concentrations the percentage decrease in yield is greater. By carrying out the reaction in vessels evacuated to 20 to 30 mm., as is done in the gasometric ninhydrin method (13), the color yield from 2.0 mM leucine solutions can be raised almost to the maximum value. At a leucine concentration of 0.05 mM, however, the results still run about 10 per cent low.

The first trials on the blocking of the oxidative side reaction by the addition of a reducing agent to the ninhydrin solution were made with hydrindantin. Consistent results were obtained at a hydrindantin concentration of 1 mg. per cc. The color yields were unaltered by a 4-fold increase in this concentration. Since hydrindantin can be prepared by the action of stannous chloride on ninhydrin, the addition of stannous chloride directly to the reagent solution was tried and found to give the same results. However, the presence of hydrindantin, which is highly insoluble in water, required the addition of an organic solvent which would keep this compound in solution during the course of the reaction and during storage of the reagent solution. Among the solvents tested, methyl cellosolve had the highest solvent power for hydrindantin. The solvent mixture chosen (1:1 water-methyl cellosolve) does not evaporate in the water bath at 100° and does not precipitate sodium citrate from the buffer.

SUMMARY

The reaction of ninhydrin with NH_2 groups to give diketohydrindylidene-diketohydrindamine has been utilized as the basis for a photometric determination of amino acids and related compounds in effluent samples from starch chromatograms. The color yields have been rendered fully reproducible by the incorporation of hydrindantin or stannous chloride in the reagent solution to eliminate oxidative side reactions. Although the color yield from a given amino acid is constant, the different amino acids do not all give the same percentage yield of the blue product. This fact does not prevent the accurate use of the method in chromatographic work in those cases in which the individual amino acids are separated from one another by the fractionation process.

Color development is obtained with a variety of compounds containing NH_2 groups, including amino acids, peptides, primary amines, and ammonia. For chromatographic work, the generality of the reaction extends its usefulness. For work with unfractionated biological material, the lack of specificity is a disadvantage.

The reaction is carried out at pH 5 and 100°. The absorption maximum of the blue product is at 570 mμ. On individual amino acids the accuracy is 2 per cent for samples in the range of 2.5 γ of α-NH_2 nitrogen. The mechanics of the procedure have been developed to permit the analysis of a large number of samples on a routine basis.

The authors wish to acknowledge the assistance of Miss Enid Mellquist and Mr. Anton Hornicek in the performance of this work.

BIBLIOGRAPHY

1. Stein, W. H., and Moore, S., *J. Biol. Chem.*, **176**, 337 (1948).
2. Harding, V. J., and MacLean, R. M., *J. Biol. Chem.*, **20**, 217 (1915); **25**, 337 (1916).
3. Harding, V. J., and Warneford, F. H. S., *J. Biol. Chem.*, **25**, 319 (1916).
4. Riffart, H., *Biochem. Z.*, **131**, 78 (1922).
5. Cherbuliez, E., and Herzenstein, A., *Helv. chim. acta*, **17**, 1440 (1934).
6. Polonovski, M., and Moreno-Martin, F., *Compt. rend. Soc. biol.*, **119**, 583 (1935).

7. Virtanen, A. I., and Laine, T., *Skand. Arch. Physiol.*, **80**, 392 (1938).
8. Wieland, T., and Wirth, L., *Ber. chem. Ges.*, **76 B**, 823 (1943).
9. Ruhemann, S., *J. Chem. Soc.*, **99**, 792, 1306, 1486 (1911).
10. Tetzner, E., *Mikrochemie*, **28**, 141 (1940).
11. Koppanyi, T., Vivino, A. E., and Veitch, F. P., Jr., *Science*, **101**, 541 (1945).
12. Grassmann, W., and von Arnim, K., *Ann. Chem.*, **509**, 288 (1934).
13. Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 627 (1941).
14. Frame, E. G., Russell, J. A., and Wilhelmi, A. E., *J. Biol. Chem.*, **149**, 255 (1943). Russell, J. A., *J. Biol. Chem.*, **156**, 467 (1944).
15. Chinard, F. P., and Van Slyke, D. D., *J. Biol. Chem.*, **169**, 571 (1947).
16. Teeters, W. O., and Shriner, R. L., *J. Am. Chem. Soc.*, **55**, 3026 (1933).

OXIDATION OF GLUCOSE LABELED WITH RADIOACTIVE CARBON BY NORMAL AND ALLOXAN-DIABETIC RATS*

BY D. B. ZILVERSMIT,† I. L. CHAIKOFF, D. D. FELLER,‡ AND E. J. MASORO

(*From the Division of Physiology, University of California Medical School, Berkeley*)

(Received for publication, June 23, 1948)

The nature of the metabolic defect in diabetes has been vigorously debated for well over 30 years, but despite the interesting new lights shed on it during the past few years (1, 2) *rigid* proof as to whether the derangement in this disease results from an impaired capacity of the diabetic animal to convert glucose to CO_2 is still lacking. With the introduction of isotopic carbon, it became possible for the first time to study the direct conversion of carbon-containing compounds to CO_2. We wish to report here observations dealing with the fate of the carbon of administered radioglucose in normal and alloxan-diabetic rats. The evidence obtained indicates that the over-all rate of oxidation of glucose by the alloxan-diabetic rat need not differ significantly from that in the normal rat.

EXPERIMENTAL

Production and Care of Diabetic Rats—The first five rats (Table I) were injected intraperitoneally with a 2 per cent aqueous solution of alloxan. A single injection of 200 mg. per kilo of body weight was found effective in producing glycosuria and at the same time kept mortality at a minimum. However, the intraperitoneal route of administration produced adhesions in the abdominal cavity, and for this reason the alloxan was injected intravenously in the other animals. A dose of 50 mg. of alloxan per kilo of body weight, administered as a 5 per cent solution in isotonic saline, was used for intravenous purposes. Higher dosages resulted in undue mortality, whereas lower doses frequently failed to induce diabetes.

The animals were kept in individual cages in a warm room. The stock diet (which consisted of 68.5 parts of wheat, 5 of casein, 10 of fish meal, 1.5 of salt, 5 of a fish oil, and 10 of alfalfa) was supplemented twice weekly with lettuce.

Urine was collected daily and preserved with toluene. Glucose in urine was determined on the day of collection by oxidation with potassium ferri-

---

* Aided by grants from the American Cancer Society (recommended by the Committee on Growth of the National Research Council) and the Sugar Research Foundation, Inc.

† Fellow of the American Cancer Society.

‡ United States Public Health Fellow.

cyanide and subsequent titration with ceric sulfate (3, 4). Blood glucose was determined by the same method on an aliquot of a protein-free filtrate prepared with $ZnSO_4$ and NaOH (5). The last traces of zinc were removed by the addition of sodium carbonate.[1]

The duration of diabetes, degree of glycosuria, and weight changes that occurred in the nine diabetic rats used in this study are recorded in Table I.

Preparation of C^{14}-Labeled Glucose[2]—C^{14}-labeled starch and glucose were isolated from tobacco leaves which had been illuminated in the presence of $C^{14}O_2$. Labeled glucose was then obtained by acid hydrolysis of the starch and by fractional crystallization of the soluble sugars as described by Putman *et al.* (6). The purified radioglucose was dissolved in an isotonic NaCl solution and kept frozen (−18°) until just before it was injected.

TABLE I

History of Diabetic Rats

Rat No.	Route of alloxan administration	Duration of diabetes before experiment	Sugar excretion			Weight			Average volume of urine per day
			Maximum per 24 hrs.	During experiment		Before alloxan injection	On day of experiment	Minimum during diabetic state	
		days	*gm.*	*hrs.*	*gm.*	*gm.*	*gm.*	*gm.*	*cc.*
D3	Intraperitoneal	22	6.8	6	1.13	195	158	124	60
D6	"	16	5.6	6	0.36	150	150	132	50
D20	"	10	10	48	18.2	300	270	228	120
D23	"	48	11	12	2.45		163	160	70
D28	"	70	7.6		7.6*	218	212	208	50
D40	Intravenous	14	8.4	12	4.20	190	174	158	50
D48	"	25	6.8		6.6*	168	160	152	60
D53	"	20	11.3		11.3*	262	186	186	50
DJ2	"	14	11.4	46	7.0	200	164	129	65

* Rats so designated were nephrectomized; the amounts recorded were excreted during the 24 hours preceding nephrectomy.

Collection and Determination of Exhaled CO_2—During the experiment the rat was kept in an all-glass cage which was ventilated continuously with CO_2-free air at 27–28°. The air collected from the cage was passed through a column of carbonate-free NaOH (40 milliequivalents were used for each hour of CO_2 collection per rat). A porous glass disk at the bottom of the column served to break the stream of air into fine bubbles. The apparatus was tested for complete recovery of the expired CO_2. The rats had access to food and water at all times while in the cage.

---

[1] Kaplan, A., unpublished observations.

[2] We are indebted to Dr. W. Z. Hassid for the samples of photosynthetically prepared radioglucose used in this study.

The NaOH-Na_2CO_3 solution was made to volume and the amount of carbonate determined by titration of two different aliquots with 0.1 N HCl. One aliquot was titrated to a brom-cresol green end-point. The value so obtained is a measure of the amounts of NaOH and Na_2CO_3 present. To the other aliquot an excess of $BaCl_2$ was added before titrating it to the phenolphthalein end-point; this titration value represents the amount of unused NaOH. The difference between the two titration values, therefore, gives the amount of Na_2CO_3 that was formed during the collection of CO_2. After centrifugation, the $BaCO_3$ precipitate was washed twice with distilled water and suspended in alcohol. The precipitate was ground in a glass homogenizing tube, mounted on an aluminum disk, and its radioactivity determined after the manner described by Dauben *et al.* (7). In general, each sample was counted for more than 3000 counts; the error of the counting was less than 2 per cent.

Determination of Radioglucose in Urine. Fermentation—A sample of urine buffered with phosphate at about pH 5 was incubated at 37° with yeast (*Torula monosa*). The yield of CO_2 evolved was improved by the presence of 10^{-3} M sodium azide (8). The carbon dioxide was collected in 0.25 N barium hydroxide solution containing 2 per cent $BaCl_2$ according to Van Slyke and Folch (9). The precipitate of barium carbonate which formed was treated as described for respiratory CO_2. The total amount of C^{14}-labeled glucose excreted in urine was obtained from the specific activity of its barium carbonate precipitate and the total urinary glucose from its reducing value.

The validity of the fermentation procedure was tested on a sample of 3,4-C^{14}-labeled glucose. This glucose was prepared by a modification of the method of Solomon *et al.* (10) as follows. A 200 gm. rat was fasted for 24 hours. 340 mg. of Na lactate[3] were administered by stomach tube. 1 hour later the animal was injected intraperitoneally with 1 millicurie of $NaHC^{14}O_3$ contained in isotonic $NaHCO_3$. 2 hours after the administration of the labeled $NaHCO_3$ the animal was sacrificed and glycogen isolated from its liver (13). Glucose was obtained by hydrolyzing the isolated glycogen for 3 hours with 0.6 N HCl.

According to Wood *et al.* (14) all the C^{14} atoms in this glucose are in the 3 and 4 positions. Our finding that the specific activity of the $C^{14}O_2$ produced by fermentation was 3 times as high as that of the CO_2 when the whole glucose molecule was oxidized seems adequate evidence that the fermentation with *Torula monosa* is a reliable method for the determination of the specific activity of both carbon atoms 3 and 4 of the glucose molecule.

---

[3] The amount of lactate administered was based on earlier observations of Cori and Cori dealing with liver glycogen formation from lactic acid (11). It was found in the laboratory that during the first 6 hours glycogen is deposited at a uniform rate in the liver of the 24 hour fasted rat fed lactate. Liver glycogen was determined by the method of van Wagtendonk (12).

Isolation of Osazone—The glucose content of a sample of urine was determined, and for each gm. of glucose present 10 gm. of phenylhydrazine hydrochloride and 15 gm. of sodium acetate trihydrate were added. The mixture was heated on a steam bath for 1.5 hours and then kept at 4° for approximately 12 hours. The mixture was centrifuged, and the osazone crystals obtained were washed several times with distilled water. The crystals were next dissolved in hot 50 per cent alcohol, and insoluble materials present removed by filtration through a steam jacket Büchner funnel. The glucosazones were allowed to crystallize at 4° and then dried in a vacuum desiccator over $CaCl_2$.

The glucosazones were oxidized by heating them with a chromic-sulfuric acid mixture, according to Van Slyke and Folch (9), and the carbon dioxide evolved was trapped in a mixture of 0.25 N barium hydroxide and 2 per cent barium chloride and treated as described above. 92 to 93 per cent of the osazone carbon was recovered as barium carbonate. The barium carbonate was mounted on an aluminum disk as described above, and its radioactivity measured. The measurement of the radioactivity of the glucose is much more tedious by this method than by the fermentation procedure. Furthermore, the counts per mg. of $BaCO_3$ obtained by the osazone procedure suffer a 3-fold dilution by the phenylhydrazine carbon.

Tables III and IV show that the values obtained for radioglucose by the two methods (osazone and fermentation) are in fairly good agreement. This agreement implies that the specific activity of the 3rd and 4th carbons of the glucose molecule is approximately equal to that of the other carbons. This is important since it excludes the possibility that the expired $C^{14}O_2$ is derived only from one or two specific carbons of the glucose chain.

Results

Conversion of Radioglucose to CO_2 by Normal and Diabetic Rats

Normal Rats—Rats N1 and N2 received intraperitoneally 100 mg. of radioglucose (Table II), whereas Rats 3 to 5 were injected intraperitoneally with 1 gm. of labeled glucose per kilo of body weight. The amounts of radioactive CO_2 recovered in the expired air of each rat are recorded in Table II. During the first 4 hours, the administered labeled glucose was rapidly oxidized by the normal rat. By the time 6 hours had elapsed, 40 to 60 per cent of the administered C^{14} was recovered in the expired CO_2. Although in 6 hours Rat N2 eliminated as CO_2 as much as 55 per cent of the radioglucose that it received, only an additional 11 per cent was recovered as expired radioactive CO_2 during the next 18 hours. The more rapid conversion of the administered labeled glucose to CO_2 during the early intervals means, of course, that at these times the animal's labeled glucose pool had a high specific activity and that at the later intervals this had been diluted by unlabeled glucose derived either from the diet or from endogenous sources.

Diabetic Rats—At the time radioactive glucose was injected, diabetes had existed in the animals used for periods varying from 10 to 48 days (Table I). The rats displayed such manifestations of diabetes as polyuria, glycosuria, polyphagia, and weight loss. Each rat excreted from 50 to 120 cc. of urine per day. The amount of glucose excreted was, of course, not constant from day to day. The maximum found before the administration of radioglucose amounted to 11 gm. per 24 hours.

TABLE II

*Oxidation of Intraperitoneally Injected Radioglucose to CO_2 by Normal Rats**

Rat No.	Weight	Radioglucose injected intraperitoneally	Per cent of administered glucose† converted to CO_2 at end of				
			2 hrs.	4 hrs.	6 hrs.	12 hrs.	24 hrs.
	gm.	*mg.*					
N1	172	100			56		
N2	250	100	27.6	48.4	55	61.6	65.7
N3	230	230‡			56.7	63.3	
N4	270	270‡			45.9	53.6	
N5	280	280‡			39.4	49.6	

* The rats had access to food throughout the period of observation.

† The administered glucose contained 1 to 2 million counts per minute.

‡ Equivalent to 1 gm. per kilo of body weight.

In contrast to the normal rat, which had converted approximately 40 to 60 per cent of the administered glucose to CO_2 in 6 hours, the five diabetic rats used in this study (Tables III and IV) oxidized only 11 to 24 per cent of the injected labeled glucose in 6 hours. An examination of the urine revealed, however, that most of the administered radioglucose was not available for oxidation but had been excreted. Thus Rat D20 excreted more than 60 per cent of the administered glucose in the first 12 hours after its injection (Table III). Little more radioglucose was excreted in the next 36 hours. This does not mean that the rat did not continue excreting glucose, but rather that after 12 hours the specific activity of the body glucose had dropped to low levels. In 6 hours the other four rats (Table IV) excreted from 30 to 45 per cent of the labeled glucose they had received intraperitoneally.

Calculation of Rate of Glucose Oxidation

From the amount of $C^{14}O_2$ expired during a given interval after the injection of the radioglucose, it is possible to obtain an approximate value for the amount of plasma glucose that had been oxidized during that interval.

TABLE III

Oxidation of 100 Mg. of Intraperitoneally Injected Radioglucose by Diabetic Rat D20

Interval	Total glucose	Radioglucose, per cent of injected dose determined by		Specific activity* of urinary glucose $\times 10^3$	Expired $C^{14}O_2$, per cent of administered glucose†	Glucose oxidized
		Fermentation	Osazone			
hrs.	*mg.*					*mg. per hr.*
0- 2	775	49.3	43.1	60	6.6	55
2- 4	513	11.0	9.7	20	3.3	82
4- 6					1.5	
4-12	2,260	3.3	5.0	1.9		
6-12					2.1	
12-24	5,350	0.7‡	‡	0.13	1.0	
24-48	9,300	‡	‡		0.7‡	
Total.....	18,200	64.3			15.2	

* The specific activity is expressed as the per cent of the injected C^{14} per mg. of glucose. The values are based on averages between fermentation and osazone.

† The injected dose contained 1,800,000 counts per minute.

‡ The sample counted less than 1.2 × background.

TABLE IV

Oxidation of Intraperitoneally Injected Glucose by Diabetic Rats D3, D6, D23, and D40

Rat No.	Weight	Radio-glucose injected	Urinary glucose								Expired $C^{14}O_2$, per cent of administered glucose*		Glucose oxidized‡
			Total		Radioglucose, per cent of injected dose* determined by				Specific activity† of urinary glucose $\times 10^3$				
					Fermentation		Osazone						
			1st 6 hrs.	2nd 6 hrs.	1st 6 hrs.	2nd 6 hrs.	1st 6 hrs.	2nd 6 hrs.	1st 6 hrs.	2nd 6 hrs.	1st 6 hrs.	2nd 6 hrs.	
	gm.	*mg.*	*mg.*	*mg.*									*mg. per hr.*
D3	158	100	1130		34		36.8		31		23		122
D6	150	100	360		46		44.5		125		24		32
D23	163	163§	379	2070	30	13.5	30.5	10.9	79	6.0	15	3.7	32
D40	174	174§	1360	2840			45.7	3.0	34	1.1	17.5	1.8	87

* The administered glucose contained 1 to 2 million counts per minute.

† The specific activity is expressed as the per cent of the administered C^{14} per mg. of glucose.

‡ These values are determined from the specific activity of the urinary glucose and the expired $C^{14}O_2$ during the first 6 hours of the experiment.

§ The glucose injected equals 1 gm. per kilo of body weight.

To make such a calculation, we used the *average* specific activity of plasma glucose during the period under consideration. But we have to be reasonably certain that the expired $C^{14}O_2$ is derived only from labeled glucose; as a rule, this will be true only during the early intervals after the injection of radioglucose when little or none of it has been converted to other compounds.

To determine the specific activity of plasma glucose, an aliquot of the plasma sample was oxidized and its total radioactivity determined as $BaCO_3$, whereas on a different aliquot of this same plasma the glucose content was determined from its reducing value. In the diabetic animals we were able to check this procedure by demonstrating that the specific activity so obtained was equal to the specific activity of the urinary glucose. This is shown in Fig. 1.

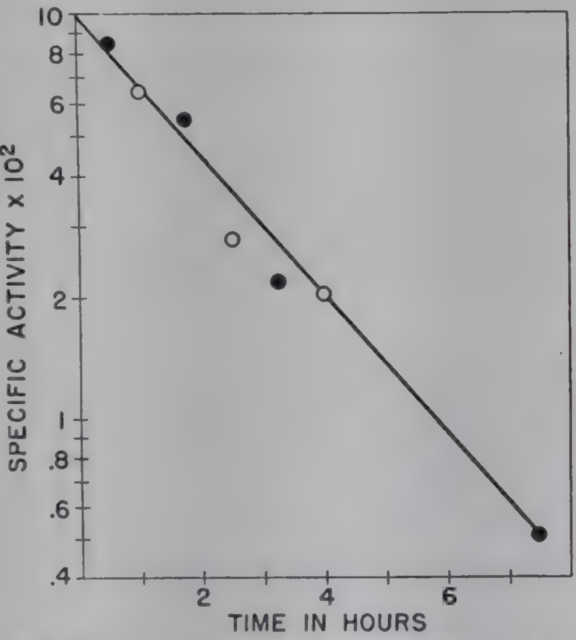


FIG. 1. Changes with time in specific activity of urinary and plasma glucose of Rat DJ2. The ordinate scale is logarithmic. ○ plasma glucose; ● urine glucose.

Values for specific activities of plasma and urinary glucose of two diabetic rats (Rats DJ2 and D20) are presented in Figs. 1 and 2; here the values are plotted against time on semilog paper. This plot was found to be a straight line during the first 6 hours (15). During the interval from 0 to 2.5 hours the average specific activity of plasma glucose, expressed as a percentage of the injected dose per mg. of glucose, is approximately 6×10^{-2} (see Fig. 1). The amount of $C^{14}O_2$ exhaled during that interval was approximately 6 per cent of the administered radioactivity. Therefore, the amount of glucose oxidized in this period is equal to $6/(6 \times 10^{-2}) = 100$ mg. or 40 mg. per hour. The same calculation for the interval from 2.5 to 4 hours, in which 2 per cent of the injected dose was exhaled, gives an oxidation of 50 mg. of glucose per hour.

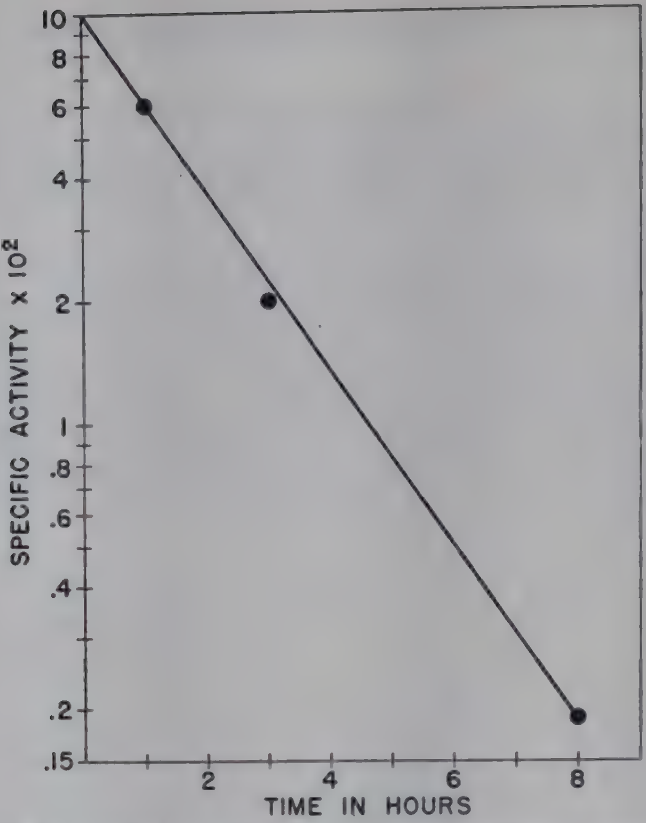


FIG. 2. Changes with time in specific activity of urinary glucose of Rat D20 (see Table III).

TABLE V

Rate of Oxidation of Glucose by Normal Rats

Each rat was injected intraperitoneally with 15 mg. of labeled glucose.

Rat No.	Weight	$C^{14}O_2$ collected		Plasma glucose		Glucose oxidized
		Interval	Per cent of injected dose*	Time after injection	Specific activity† $\times 10^2$	
	gm.	*hrs.*		*hrs.*		*mg. per hr.*
NJ1	220	0–1	13.6		‡	50
		1–2	10.6	1	20.8	66
		2–4	5.5	3	6.9	40
		4–6	1.84	6	4.6	
NJ2	242	0–1	8.75		‡	32
		1–2	9.0	1	19	69
		2–4	5.25	3	4.1	64
		4–6	2.73	6	4.1	
NJ3	250	0–1	7.93		‡	28
		1–2	14.1	1	21.8	85
		2–3	4.63	3	7.6	48

* The injected glucose contained 1,600,000 counts per minute.

† The specific activity is expressed as the percentage of the injected C^{14} per mg. of glucose.

‡ The average specific activity of plasma glucose was obtained by extrapolation (see the text).

The values obtained by this type of calculation for the amounts of glucose oxidized per hour by normal rats are recorded in Table V and by diabetic rats in Tables III and IV. The range of values calculated for the normal rat was similar to that found for the diabetic.

Comparison of Ability of Nephrectomized Normal and Nephrectomized Alloxan-Diabetic Rats to Convert Radioglucose to CO_2

Since the diabetic rat excreted a good portion of the administered glucose in the urine, the amount of $C^{14}O_2$ exhaled, as noted above, provided no basis for comparing the capacities of normal and diabetic rats to oxidize administered glucose. To prevent loss of glucose by this route, radioglucose was injected intravenously into rats 15 minutes after they had been subjected to bilateral nephrectomy. Each rat received 1 gm. of glucose per kilo of body weight. This dose was selected because it was large enough to yield similar *initial* values for the specific activities of circulating glucose in both normal and diabetic rats. In other words, this dose minimized the differences in plasma glucose between the normal and the diabetic rats at the very beginning of the experiment. Data on the conversion of injected radioglucose to $C^{14}O_2$ by the two types of rats under these conditions are recorded in Table VI.

TABLE VI

Conversion of Intravenously Injected Radioglucose to CO_2 by Nephrectomized Normal and Nephrectomized Alloxan-Diabetic Rats

Each rat received 1 gm. of labeled glucose per kilo of body weight.

Rat No.	Weight	Per cent of administered glucose* recovered as CO_2 for each interval						Blood sugar at end of experiment
		0-0.5 hr.	0.5-1 hr.	1-2 hrs.	2-4 hrs.	4-6 hrs.	Entire 6 hrs.	
	gm.							*mg. per cent*
Normal. N6	180	5.7	5.3	8.0	14.0	3.9	36.9	131
" N7	172	2.7	4.7	6.0	12.3	3.9	29.6	157
" N8	202	3.9	4.4	8.7	15.6	3.3	35.9	125
" N9	174	5.6	4.8	8.5	16.7	3.6	39.2	138
Diabetic. D28	212	0.42	1.4	4.5	10.5	9.4	26.2	700
" D48	160	2.2	5.9	10.0	13.7	7.8	39.6	870
" D53	186	0.57	2.1	4.3	11.4	7.5	25.9	1060

* The administered glucose contained 1 to 2 million counts per minute.

Judging from the $C^{14}O_2$ exhaled during the entire 6 hours, there appears to be no significant difference between nephrectomized normal and nephrectomized alloxan-diabetic rats in their ability to oxidize the administered glucose. It is interesting to note, however, that in the early intervals two of the three diabetic rats exhaled less $C^{14}O_2$ than the normal ones, whereas at the later intervals (4 to 6 hours) the reverse was the case.

6 hours after the administration of the large dose of labeled glucose, namely 1 gm. per kilo of body weight, the plasma glucose in the nephrectomized normal rats had returned to normal levels or levels close to normal; the highest value observed at the 6 hour interval was 160 mg. per cent. In the diabetic rats, on the other hand, plasma glucose of 700 to 1000 mg. per cent was found when they were sacrificed.

DISCUSSION

Normal rats injected intraperitoneally or intravenously with amounts of glucose varying from 100 to 280 mg. converted 40 to 50 per cent of it to CO_2 in 6 hours. In the nephrectomized but otherwise normal rat, $C^{14}O_2$ appeared in the exhaled gas within the first half hour, and at the end of the 1st hour nearly 10 per cent of the intravenously injected glucose was converted to CO_2. These findings are in agreement with the classical view that administered glucose is available for immediate oxidation by the animal body.

In order to compare the rates of *conversion* of *plasma* glucose to CO_2 by normal and diabetic rats, two methods of study were employed: one was based on the $C^{14}O_2$ evolved when a relatively large amount of labeled glucose was introduced intravenously into nephrectomized preparation; in the second the amount of glucose oxidized in the intact rat was derived from measurements of the $C^{14}O_2$ evolved and of the specific activity of plasma glucose. That the alloxan-diabetic rat can oxidize appreciable amounts of glucose cannot be doubted from the results obtained by the first method. As judged by the $C^{14}O_2$ eliminated during an entire 6 hour period of observation, the amounts of administered glucose oxidized by the nephrectomized normal and nephrectomized diabetic rats are of the same order. *The values for glucose oxidation obtained by the second method refer specifically to the conversion of plasma glucose (and such glucose as exists in rapid equilibrium with plasma glucose) to CO_2.*

The interpretation of our findings is not without some difficulty. The values for specific activity of plasma glucose are based on two measurements; namely, (1) the total radioactivity of plasma, which we have assumed to be glucose at the early intervals, and (2) the total reducing value of plasma, which we have also assumed to be glucose. The reliability of the specific activity measurements for plasma glucose is shown by the finding that in the diabetic rat the values obtained agreed with those found for the osazones prepared from urinary glucose. In the normal rat it was not possible to make such a comparison. The assumptions made in connection with the specific activity would appear, however, to be well supported by our observations in the nephrectomized rats; the finding that the over-all capacity of the normal nephrectomized rat to oxidize glucose is of the same order as that of the diabetic involves none of the assumptions referred to above.

An interference was observed in the oxidation of the injected carbohydrate by the nephrectomized diabetic rat during the 1st hour, whereas at the later intervals the amount of $C^{14}O_2$ produced by these rats was of the same order of magnitude as that produced by normal rats. The interpretation of the results of the early intervals presents some difficulties. This phase is being investigated further.

In order to interpret the $C^{14}O_2$ data presented here for the alloxan-treated rats, the degree of diabetes that existed in these rats must be evaluated. Postmortem examination of their pancreases revealed massive necrosis of the islets. It cannot be inferred, however, that the alloxan-diabetic rat is deprived of *all* insulin-secreting tissue. But even though the presence of some residual insulin cannot be excluded in the diabetic rats used in this study, they nevertheless excreted, when fed, from 5 to 11 gm. of glucose daily and exhibited such manifestations of diabetes as polyphagia, polyuria, polydipsia, and loss of weight. Another indication of the degree of diabetes in the rats used here is provided by the finding of values for plasma glucose of 700 to 1000 mg. per cent 6 hours after excision of their kidneys.

SUMMARY

1. The rate of conversion of administered radioglucose to CO_2 was measured in normal and alloxan-diabetic rats.

2. $C^{14}O_2$ appeared within the first 30 minutes in the expired air of both normal and alloxan-diabetic rats.

3. The amount of *plasma* glucose converted to CO_2 was measured by two methods. The first was based on the specific activities of plasma glucose and the amounts of $C^{14}O_2$ in the expired air, the second on the amounts of $C^{14}O_2$ exhaled by the nephrectomized rat. As judged by these procedures, the rate of conversion of *plasma glucose* to CO_2 by the alloxan-diabetic rat does not differ significantly from that found in the normal.

BIBLIOGRAPHY

1. Cori, C. F., *Harvey Lectures*, **41**, 253 (1945–46).
2. Stetten, D., *J. Am. Med. Assn.*, **132**, 373 (1946).
3. Hassid, W. Z., *Ind. and Eng. Chem., Anal. Ed.*, **8**, 138 (1936).
4. Miller, B. F., and Van Slyke, D. D., *J. Biol. Chem.*, **114**, 583 (1936).
5. Somogyi, M., *J. Biol. Chem.*, **86**, 655 (1930).
6. Putman, E. W., Hassid, W. Z., Krotkov, G., and Barker, H. A., *J. Biol. Chem.*, **173**, 785 (1948).
7. Dauben, W. G., Reid, J. C., and Yankwich, P. E., *Anal. Chem.*, **19**, 828 (1947).

8. Winzler, R. J., *Science*, **99**, 327 (1944).
9. Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, **136**, 509 (1940).
10. Solomon, A. K., Vennesland, B., Klemperer, F. W., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, **140**, 171 (1941).
11. Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, **81**, 389 (1929).
12. van Wagtendonk, W. J., Simonsen, D. H., and Hackett, P. L., *J. Biol. Chem.*, **163**, 301 (1946).
13. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, **100**, 485 (1933).
14. Wood, H. G., Lifson, N., and Lorber, V., *J. Biol. Chem.*, **159**, 475 (1945).
15. Zilversmit, D. B., Entenman, C., Fishler, M. C., and Chaikoff, I. L., *J. Gen. Physiol.*, **26**, 333 (1943).

PHYSICOCHEMICAL PROPERTIES OF CRYSTALLINE CLOSTRIDIUM BOTULINUM TYPE A TOXIN*

BY FRANK W. PUTNAM,† CARL LAMANNA, AND D. G. SHARP

(From Camp Detrick, Frederick, Maryland, and the Department of Surgery, Duke University School of Medicine, Durham, North Carolina)

(Received for publication, June 25, 1948)

Interest in the chemical nature and mode of action of bacterial toxins has been renewed by the simultaneously announced crystallization of botulinal (1) and tetanal toxins (2) and the recent purification of several toxoids (3–5). Of these, crystalline *Clostridium botulinum* type A toxin alone has been submitted to an apparently complete elementary and amino acid analysis (6), as well as to preliminary physicochemical characterization (7–9) and immunological study (10). This toxin has been found to be a typical protein exhibiting no unique composition or physicochemical properties and is apparently devoid of a prosthetic group. A preliminary report of the molecular weight and homogeneity of botulinal toxin prepared by the method of Lamanna *et al.* (1) has already been made (9). Further details of the physicochemical characterization of this toxin and of the effect of the method of preparation on the molecular kinetic and serological properties of this substance are given in this report. Investigation of the botulinal toxin-antitoxin reaction has also been undertaken. Data to be published separately indicate that serologically the toxin acts as a single substance (10).

The original procedure for the crystallization of botulinal toxin (1) has been modified in several ways, including the omission of a step involving shaking of the crude acid-precipitated toxin with chloroform (11). However, much study had already been made of the properties of crystalline toxin of maximum potency prepared by the procedure first announced. A later method described by Abrams, Kegeles, and Hottle (7) differs from both the above procedures and also omits shaking with chloroform. In view of the suggestion (8) that partial denaturation may result from the use of chloroform in the purification of the toxin, some data on the physicochemical and serological characteristics of toxin prepared both by the original and the modified methods of Lamanna *et al.* are included in this communication.

---

* Some of the material in this paper was presented at the meeting of the Federation of American Societies for Experimental Biology at Chicago, May, 1947 (*Federation Proc.*, **6**, 284 (1947)).

† Present address, Department of Biochemistry, University of Chicago.

EXPERIMENTAL

The crystalline toxin used in this investigation was prepared by methods previously described (1, 11). Some of the material was later used for the amino acid analysis already mentioned (6). Several lots were studied, including one not subjected to shaking with chloroform (Batch S-5). The potency of the toxin varied somewhat from preparation to preparation but probably not significantly outside the errors of the mouse titration employed. The toxin contained about 220 × 10^6 mouse LD_{50} per mg. of N as measured by a statistically valid titration (12) on 20 gm. white mice. The highest value recorded was 250 × 10^6 LD_{50} per mg. of N (initial potency of Batch C). A single batch of toxin (Batch BT-3), characterized successively in electrophoresis, diffusion, and sedimentation behavior, retained a potency of 170 × 10^6 LD_{50} per mg. of N upon recovery. It was observed, however, that even mild physical treatment of the purified toxin resulted in some loss of toxicity, especially at high dilutions.

Electrophoresis

The electrophoretic homogeneity of botulinal toxin was studied with the aid of the Tiselius apparatus (13). One apparatus used was equipped with the Philpot-Svensson optical system (14); another employed in later experiments utilized the Longsworth scanning system (15). All physical measurements were made on preparations equilibrated by long dialysis against buffer. All pH measurements were made with the glass electrode.

Toxin of maximum potency prepared by shaking with chloroform is homogeneous in electrophoresis, as is illustrated by the diagrams of Fig. 1. However, the relatively small amounts of purified toxin available restricted the scope of these experiments. In this instance, only the lower middle section and bottom section of the two-compartment cell could be filled with the protein solution. In Fig. 1, photographs[1] at two time intervals are given to illustrate the low degree of boundary spread with time. The upper diagrams represent migration for 11,400 seconds, the lower for 14,400 seconds. The mobility at 1° in 0.1 N sodium acetate buffer, pH 4.38, calculated from the diagrams of Fig. 1, is 2.75 × 10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$.

No indication of a component other than toxin is to be seen in Fig. 1. It was regularly found that toxin freshly crystallized by the original method of Lamanna and coworkers (1) was electrophoretically homogeneous under these conditions. Occasionally, a small, fast moving shoulder was observed on the descending boundary, but this could always be removed by repeated crystallization. On long standing, one preparation (Batch C) yielded a sharp single ascending boundary of normal mobility but a split descending boundary. However, at this time the preparation also appeared somewhat inhomogeneous, as judged both from diffusion studies and from light absorption photographs in the ultracentrifuge. On conversion to toxoid by treatment with formalin, the electrophoretic diagrams of this material were unaltered, although the mobility on the alkaline side of the isoelectric point was increased.

---

[1] The initial boundaries are slightly obscured because of light absorption by a detoxifying agent added to the external constant temperature bath.

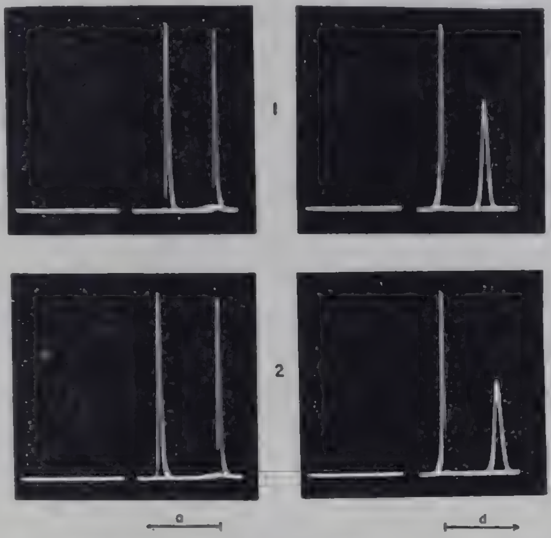


FIG. 1. Electrophoretic patterns of crystalline botulinal toxin prepared *with chloroform shaking;* pH 4.38, ionic strength 0.1, 0.1 N sodium acetate-0.2 N acetic acid buffer. Photographs taken by the Philpot-Svensson method after migration at 1° at a field strength of 5.32 volts per cm. in the two-section cell. Upper photograph, 11,400 seconds; lower photograph, 14,400 seconds. The arrow indicates the direction of migration; *a* and *d* represent the ascending and descending boundaries respectively. The very steep curves at the tails of the arrows are superimposed photographs of the starting boundaries.

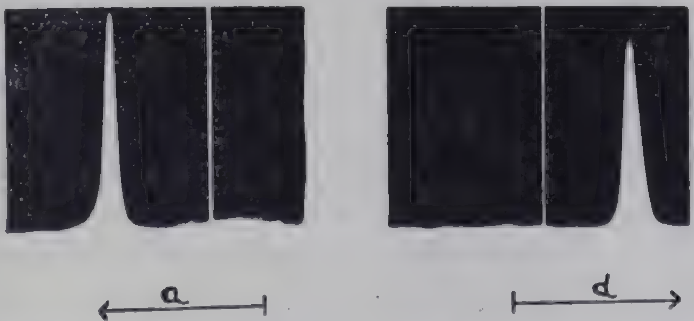


FIG. 2. Electrophoretic patterns of crystalline botulinal toxin prepared *without chloroform shaking*. Photographs taken by the Longsworth scanning method after 12,300 seconds migration at 5.37 volts per cm. Conditions and symbols as in Fig. 1.

Crystalline toxin, prepared by the modified method of Lamanna and coworkers (11) with the step of shaking with chloroform omitted, was likewise homogeneous in electrophoresis, as shown in Fig. 2. The mobility at pH 4.38 was 2.69×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$, the same within experimental error as for toxin prepared with chloroform shaking.

The small degree of boundary spread observed for both types of preparations indicated that the toxin was quite homogeneous in electrophoresis. Since there was some question that the chloroform treatment may have altered the toxin, the boundary spread for the two lots was analyzed statistically by a method already described (16). Unfortunately, the low solubility at the isoelectric point precluded boundary spread analysis in that pH range, the region best suited to this study. For a single moving boundary, the procedure of Sharp *et al.* (16) yields a value, denoted as the heterogeneity constant, which has the dimensions of mobility and increases with the electrical heterogeneity of the protein. The values for the two preparations compared favorably, being 1.4×10^{-6} cm.^{2} volt^{-1} sec.$^{-1}$ for the salt-fractionated toxin, and 1.8×10^{-6} cm.^{2} volt^{-1} sec.$^{-1}$ for toxin prepared by shaking with chloroform. These figures indicate a relatively low degree of reversible boundary spread (*i.e.*, spread not attributable to diffusion alone).

Diffusion

The diffusion constant (D) of crystalline toxin, previously found to be homogeneous in electrophoresis, was determined at 25° by the refractive index scale method of Lamm (17), in the Neurath cell and apparatus (18). The values are given in Table I. The subscripts on D indicate the several methods of calculation which allow different weight to various factors (18). For the first time interval given (84,300 seconds, or approximately 1 day) the boundary was yet too sharp for accurate measurement of the scale line displacements. With this exception, there is excellent agreement both among the values found at different time intervals and among those obtained at a given time interval when calculated by the several methods. The latter finding is a good criterion of molecular kinetic homogeneity, as measured by the somewhat insensitive method of diffusion, and may be expressed numerically by the Gralén index (19) given in the last column of Table I as $D_\sigma/D_A{}^2$. The deviation of this constant from unity is a measure of the distribution of sizes in a substance.

Similar evidence for homogeneity in diffusion behavior is offered by the low value of the standard deviation for the diffusion constant calculated at points along the curves by the method of successive analysis (the sixth column, Table I). However, the best criterion of homogeneity in diffusion lies in transforming the actual diffusion curve to normal coordinates and making graphical comparison to the ideal Gaussian distribution curve. The rather good fit obtained by this procedure and the close merging of the maximum ordinates of the ideal and the calculated curves is shown in Fig. 3 (time, 354,840 seconds).

Sedimentation in Ultracentrifuge

The sedimentation characteristics of the crystalline toxin were studied in an air-driven analytical ultracentrifuge (20), the rotor of which carried a sector-shaped cell at a mean radius of 6.5 cm. The ultraviolet light absorption method was employed for preliminary analysis, the Lamm scale method (17) for quantitative studies. Sedimentation velocity scale diagrams obtained at a mean temperature of 32° and a mean centrifugal field of 48,000*g* have already been published (9). The diagrams revealed a single sharp sedimenting boundary and yielded a value for the sedimentation constant, S_{20}, of 17.3 Svedberg units at a protein concentration of 0.17 per cent, the lowest concentration studied. Although the base-lines of the sedimentation diagrams exhibited a small angular deviation from the horizontal, planimetric measurement of the area under each boundary revealed a constant concentration of sedimenting substance equal to the initial concentration (calculation by Equation 183a (21), including a correction for the wedge-shaped nature of the cell).

TABLE I

Diffusion Constant of Crystalline Botulinal Toxin† at 25°*

Time	D_M	D_A	D_{A^2}	D_σ	D_S	$\frac{D_\sigma}{D_{A^2}}$
sec.	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	
84,300	2.08	2.10	2.12	2.19	2.05 ± 0.06‡	1.03
189,480	1.99	1.96	1.96	1.97	2.06 ± 0.07	1.00
274,860	1.96	1.95	1.95	2.01	2.05 ± 0.12	1.03
354,840	1.98	1.94	1.89	1.95	2.04 ± 0.05	1.03
Average..	$2.01 \pm 0.07 \times 10^{-7}$ cm.^{2} sec.$^{-1}$					
D_{25}.......	2.14×10^{-7} cm.^{2} sec.$^{-1}$					
D_{20}.......	1.87×10^{-7} cm.^{2} sec.$^{-1}$					

* D_M, D_A, $D_A{}^2$, D_σ, and D_S denote, respectively, the diffusion constant (with the dimensions cm.^{2} sec.$^{-1}$) calculated by the maximum height, maximum height-area (unsquared), maximum height-area (squared), standard deviation, and successive analysis methods (18). D_{25} and D_{20} denote the diffusion constant corrected to the water basis at the respective temperatures. D_{20} is given to permit comparison with the average value 2.02×10^{-7} cm.^{2} sec.$^{-1}$, determined independently in the Tiselius apparatus by the Longsworth method on other batches prepared by both groups of workers (8).

† Protein concentration of 0.63 per cent in 0.1 N sodium acetate buffer, pH 4.38.

‡ Standard deviation from the mean of determinations at six or more points along the curve.

Comparison was made also between the experimentally obtained sedimentation diagrams and the theoretical sedimentation curves which would have obtained if the boundary spreading were due solely to normal diffusion

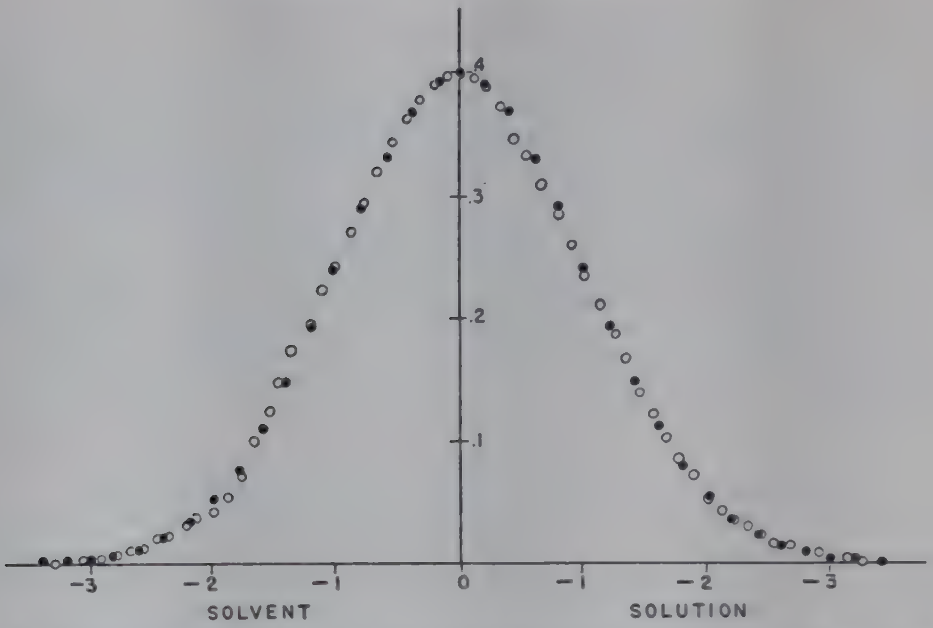


FIG. 3. Comparison of an ideal Gaussian distribution curve with the diffusion curve obtained on a 0.63 per cent solution of crystalline botulinal toxin. ● indicates the position of the ideal curve; ○ represents the experimental scale line displacements, plotted in normal coordinates. Time, 354,840 seconds.

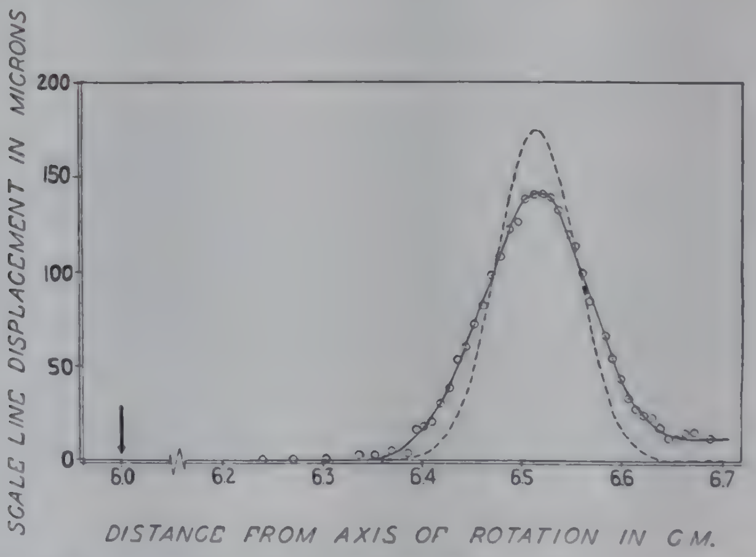


FIG. 4. Boundary spreading in a sedimentation experiment with crystalline botulinal toxin. The solid line indicates actual points obtained with the Lamm scale method; the dash line represents theoretical spreading due to diffusion alone. Time, 5400 seconds at 48,000*g*.

of the toxin. The results are shown in Fig. 4, which gives the scale diagram for Batch BT-3 after 5400 seconds sedimentation at a speed of 25,400 R.P.M. The solid line represents the actual scale diagram; the dash line depicts the calculated curve (calculation by Equation 188 (21)), with the diffusion constant obtained in separate diffusion experiments (see above). It may be seen that the boundary spread is somewhat greater than that attributable to diffusion alone. This is confirmed by the fact that the apparent diffusion constant calculated from the sedimentation diagrams, though variable, is greater than that obtained from the separate diffusion experiments.

The extraboundary blurring which occurs during the sedimentation of botulinal toxin may have been due to experimental conditions, such as possible slight vibration of the rotor or the difficulty of establishing the zero time of diffusion, or it may be indicative of a small degree of molecular heterogeneity. However, inspection of Fig. 4 reveals that the distribution of sedimentation rates does not differ significantly from the mean rate. If the increased boundary spreading is attributed to molecular heterogeneity, it could have originated either from a population of molecules differing but slightly in molecular size or from the presence of another substance of closely similar sedimentation properties. It was our experience that even mild physical procedures for study of this toxin induced some loss of potency, possibly by toxoid formation through surface denaturation. We thus attribute to this effect the small observed deviation of the actual sedimentation diagrams from theoretical curves calculated from diffusion data.

Molecular Weight and Shape

The sedimentation constant and diffusion constant taken together permit the calculation of the molecular frictional ratio $(f/f_0)_s$ according to the Perrin theory (*cf.* pp. 41–43 (21)), yielding a value of $(f/f_0)_s$ of 1.76. On the assumption that the molecules resemble prolate ellipsoids, this figure corresponds to a ratio of major to minor axis (b/a) of 14.6, hydration being neglected (21). Substitution of the sedimentation constant and the diffusion constant in the Svedberg equation (Equation 3a (21)), with a value of 0.75 (8) for the partial specific volume at 20°, yields a molecular weight of 900,000.[2]

In an effort to ascertain the particle size and shape of botulinal toxin directly, a few attempts were made to photograph a preparation shadowed with gold, by the use of the RCA electron microscope. Because of the small size of the toxin, relative to viruses and other biological substances usually studied in this apparatus, the results were inconclusive. It is hoped that resort to the newer techniques for the production of metal shadow coatings, with platinum or palladium on glass with subsequent replica stripping, will yield more decisive results in the near future.

---

[2] The partial specific volume of chloroform-fractionated botulinal toxin calculated from the complete amino acid data is 0.736. This figure based on tentative values for the constituent amino acid molar volumes leads to a slightly lower molecular weight. (Cohn, E. J., and Edsall, J. T., Proteins, amino acids and peptides, American Chemical Society monograph series, New York, 370-375 (1943).)

Immunological Homogeneity

In addition to meeting the physicochemical criteria of homogeneity in electrophoresis and diffusion and sedimenting with a single sharp boundary in the ultracentrifuge, botulinal toxin has proved to be homogeneous in immunological behavior, for both toxin and antitoxin are completely precipitated in the equivalence zone (10). Moreover, quantitative comparison of the behavior in the precipitin reaction of batches of toxin prepared with and without chloroform shaking reveals no difference as a result of this treatment. When the ratio of antitoxin to toxin nitrogen in the precipitate in the zones of antibody excess and equivalence is plotted against the amount of toxin nitrogen added, according to the procedure of Heidelberger and Kendall (22), the curves for toxin prepared by the two methods coincide (*cf.* Fig. 5). However, for both substances a curvilinear relation obtains, even when the ratio of antitoxin to toxin nitrogen is plotted against the square root of the added toxin concentration. Botulinal toxin thus differs in precipitin behavior from ordinary protein antigens which usually yield a linear relationship between the antibody to antigen ratio and the amount of added antigen. A more complete discussion of the characteristics of the botulinal toxin-antitoxin reaction will be published separately (10).

DISCUSSION

Crystalline *Clostridium botulinum* type A toxin meets some of the usual criteria of protein purity, *i.e.* electrophoretic homogeneity, homogeneity in diffusion, sedimentation with a single boundary, and serological homogeneity in the toxin-antitoxin reaction. However, the toxin fails to meet a more rigorous test of protein purity, namely sedimentation without boundary spreading greater than that solely attributable to normal diffusion. In addition, preliminary phase rule solubility studies indicated the presence of more than one component. The solubility studies were discontinued because surface denaturation of the toxin was readily brought about by the shaking methods used to achieve equilibrium.

The apparent slight molecular inhomogeneity of botulinal toxin is attributed to the marked lability of this protein rather than to the presence of foreign components. Even ordinary handling at room temperature readily induces surface denaturation of solutions, with the formation of opalescence or visible aggregates, requiring clarification by centrifuging at a low speed in the angle centrifuge prior to physicochemical study. Spontaneous loss of toxicity also occurs on long standing or at high dilution, necessitating the addition of protective agents at the extreme dilutions used in the estimation of biological potency. Rapid inactivation takes place in solutions above pH 7.

The lability and protein nature of highly purified bacterial toxins so far studied appears to be a general phenomenon. Crystalline tetanal toxin, originally electrophoretically homogeneous and apparently a single substance serologically, undergoes spontaneous detoxification on standing at 0° without loss in antitoxin-flocculating power but with marked alteration in phase rule solubility properties (23). Moreover, this toxin shows two components in the ultracentrifuge, one apparently an atoxic dimer. Diphtherial toxin is essentially homogeneous by molecular kinetic criteria (24) but is rapidly inactivated above pH 6.0 (5). It has been reported that electrophoretically isolated, electrically homogeneous, erythrogenic toxin

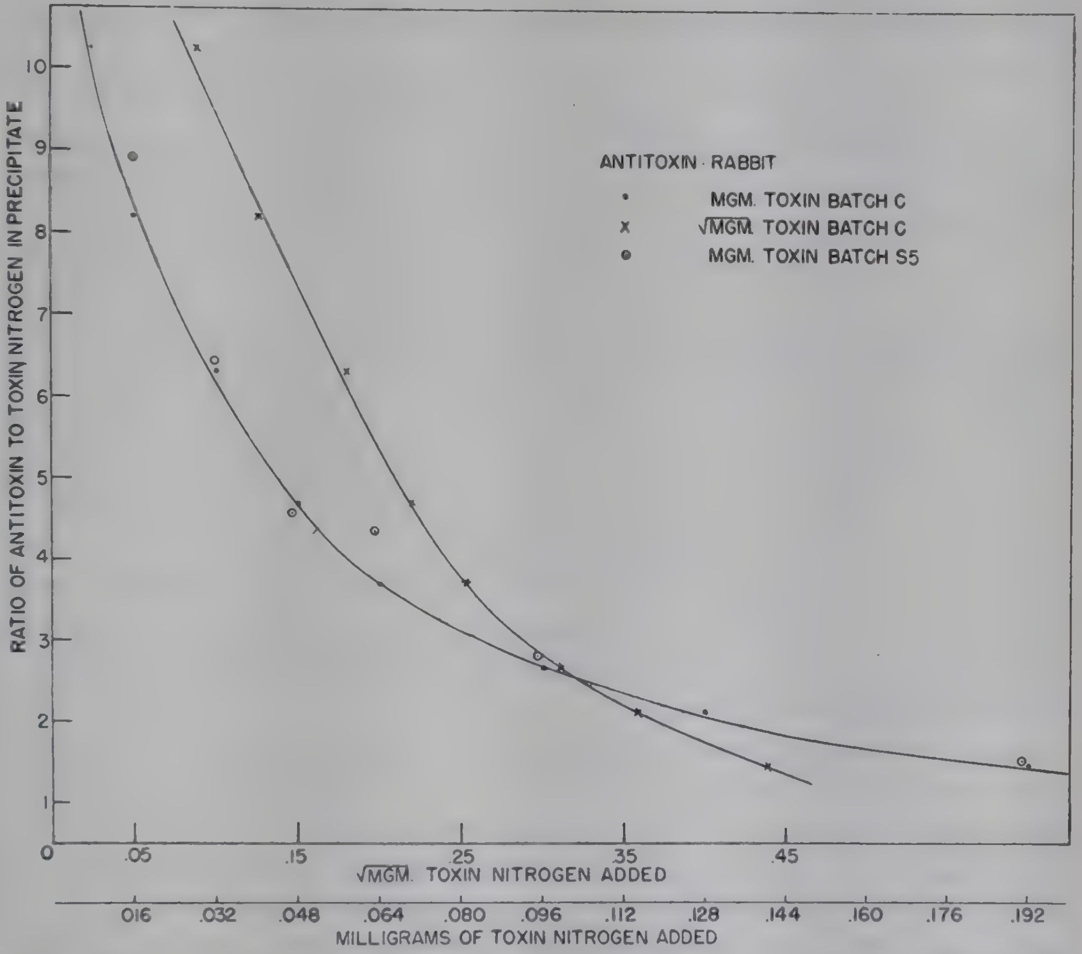


FIG. 5. Composition of toxin-antitoxin precipitates in the zones of equivalence and antitoxin excess. Batch C was crystallized by the chloroform shaking method, Batch S-5, by a different method without shaking with chloroform.

of scarlet fever is nearly homogeneous in the ultracentrifuge as judged by comparison of the actual and theoretical sedimentation curves (25). However, the diffusion constant used in the latter analysis was not obtained by special precise diffusion experiments as in this investigation, but rather was a mean value obtained by calculation from a series of the same sedimentation curves. No bacterial toxin has yet been reported to exhibit a constant solubility. However, purified diphtheria toxoid does have a constant solubility and satisfies other criteria of protein purity (5).

The molecular constants for crystalline botulinal toxins obtained in this investigation are in good agreement with those previously reported. Diffusion studies with the sintered glass disk technique afforded an estimate of about 1 million for the molecular weight (1). The minimum molecular weight calculated from amino acid analysis is approximately 45,000 (6), one-twentieth of the value, 900,000, determined by physical means and reported in this work. The molecular weight calculated by Kegeles (8) from diffusion and viscosity measurements, with the assumption that the toxin molecules approximate elongated ellipsoids, is 1,130,000. Using a different method the latter author obtained a diffusion constant for toxin prepared by the procedure of Lamanna *et al.* of $D_{20} = 1.79 \times 10^{-7}$ cm.^{2} sec.$^{-1}$, in good agreement with our value of 1.87×10^{-7} cm.^{2} sec.$^{-1}$ (correction made to 20°). However, for preparations made by the method of Abrams *et al.*, Kegeles found a mean value of $D_{20} = 2.13 \times 10^{-7}$ cm.^{2} sec.$^{-1}$. Since the ultracentrifuge and diffusion data reported in our study were obtained only on chloroform-fractionated toxin, the identity of size and shape of the toxin prepared by the several methods has not yet been clearly established.

Conclusions with regard to the apparent molecular shape of this protein must await further study. The frictional ratio derived from the sedimentation and diffusion constants in this investigation ($(f/f_0)_s = 1.76$) is markedly greater than that obtained from viscosity measurements (8) and the assumption of an elongated ellipsoidal molecular model ($(f/f_0)_\eta = 1.45$). In the latter study, fractions prepared by both methods were used in viscosity measurements, though one preparation subsequently proved to be electrophoretically inhomogeneous. Unless excessive hydration is assumed, neither value of the frictional ratio derived by these indirect physical methods is in accord with the report that electron micrographs of inactive formalin-treated toxin (toxoid) show particles nearly spherical in shape (11).

No explanation for the extreme toxicity of this protein can be deduced either from its composition or from its physical properties. However, the finding of a high molecular weight for botulinal toxin compared to other bacterial poisons poses two difficult problems: first, the explanation of the apparent absorption of the toxin through the gut in accidental botulism resulting from ingestion of spoiled foods; second, the elucidation of its mode of action on administration by either the oral or intraperitoneal route. The singular oral toxicity of this protein is in accord with reports that it is relatively resistant to the proteolytic action of pepsin and trypsin (*cf.* (26)).

The suggestion has been made that tetanal, botulinal, and diphtherial toxins may act by interfering with the synthesis of some essential enzyme, and some evidence has been presented indicating that diphtherial toxin perhaps blocks the synthesis of cytochrome *b* or some closely related enzyme (26). However, botulinal toxin is far more potent than diphtherial toxin (26), and it may be calculated that on the basis of a molecular weight of 900,000 only 20,000,000 molecules are required to kill a mouse. This fact, together with the protein nature of the toxin, suggests that it may attain its potency indirectly by acting enzymatically to synthesize a cellular poison from some normal metabolite or otherwise break an indispensable link in some physiological reaction chain in nervous tissue.

The authors are indebted to Dr. Hans Neurath of the Department of Biochemistry, Duke University School of Medicine, for making available to one of us the diffusion apparatus used in this study and to Dr. Gerson Kegeles, National Cancer Institute, for suggestions on the manuscript.

SUMMARY

1. Crystalline type A botulinal toxin is homogeneous in electrophoresis and in diffusion.

2. The toxin sediments with a single sharp boundary in the ultracentrifuge but with spreading somewhat greater than that attributable solely to diffusion. This behavior may indicate slight molecular heterogeneity attributable to the lability of this protein.

3. The molecular weight obtained from sedimentation and diffusion data is 900,000; the apparent frictional ratio is 1.76.

4. Quantitative precipitin studies indicate that botulinal toxin is serologically a single substance. The ratio of antibody to toxin in the precipitates in the zones of antibody excess and equivalence is not linearly related to the amount of antigen added.

5. Crystalline toxin prepared by several methods possesses identical electrophoretic and serological properties.

6. Its extreme potency, high molecular weight, and protein nature suggest that botulinal toxin may be an enzyme. However, no explanation of the mode of its pharmacological action is afforded by these studies.

BIBLIOGRAPHY

1. Lamanna, C., McElroy, O. E., and Eklund, H. W., *Science*, **103**, 613 (1946).
2. Pillemer, L., Wittler, R., and Grossberg, D. B., *Science*, **103**, 615 (1946).

3. Pillemer, L., *J. Immunol.*, **53,** 237 (1946).
4. Hottle, G. A., and Abrams, A., *J. Immunol.*, **55,** 183 (1947).
5. Pillemer, L., Toll, D., and Badger, S. J., *J. Biol. Chem.*, **170,** 571 (1947).
6. Buehler, H. J., Schantz, E. J., and Lamanna, C., *J. Biol. Chem.*, **169,** 295 (1947).
7. Abrams, A., Kegeles, G., and Hottle, G. A., *J. Biol. Chem.*, **164,** 63 (1946).
8. Kegeles, G., *J. Am. Chem. Soć.*, **68,** 1670 (1946).
9. Putnam, F. W., Lamanna, C., and Sharp, D. G., *J. Biol. Chem.*, **165,** 735 (1946).
10. Lamanna, C., and Doak, B. W., *J. Immunol.*, **59,** 231 (1948).
11. Lamanna, C., Eklund, H. W., and McElroy, O. E., *J. Bact.*, **52,** 1 (1946).
12. Bliss, C. I., *Ann. Appl. Biol.*, **22,** 134 (1935).
13. Tiselius, A., *Tr. Faraday Soc.*, **33,** 524 (1937).
14. Sharp, D. G., Cooper, G. R., and Neurath, H., *J. Biol. Chem.*, **142,** 203 (1942).
15. Longsworth, L. G., *Ann. New York Acad. Sc.*, **39,** 187 (1939).
16. Sharp, D. G., Hebb, M. H., Taylor, A. R., and Beard, J. W., *J. Biol. Chem.*, **142,** 217 (1942).
17. Lamm, O., *Nova acta reg. soc. sc. Upsaliensis*, **10,** No. 6 (1937).
18. Neurath, H., *Chem. Rev.*, **30,** 357 (1942).
19. Gralén, N., *Kolloid-Z.*, **95,** 188 (1941).
20. Wyckoff, R. W. G., and Lagsdin, J. B., *Rev. Scient. Instruments*, **8,** 74 (1937).
21. Svedberg, T., and Pedersen, K. O., The ultracentrifuge, Oxford (1940).
22. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, **62,** 697 (1935).
23. Pillemer, L., and Moore, D. H., *J. Biol. Chem.*, **173,** 427 (1948).
24. Pappenheimer, A. M., Jr., Lundgren, H. P., and Williams, J. W., *J. Exp. Med.*, **71,** 247 (1940).
25. Krejci, L. E., Stock, A. H., Sanigar, E. B., and Kraemer, E. O., *J. Biol. Chem.*, **142,** 785 (1942).
26. Pappenheimer, A. M., Jr., *Federation Proc.*, **6,** 479 (1947).

XANTHOMYCINS A AND B, NEW ANTIBIOTICS PRODUCED BY A SPECIES OF STREPTOMYCES*

BY C. B. THORNE† AND W. H. PETERSON

(*From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*)

(Received for publication, July 12, 1948)

Two new antibiotics have been obtained from culture filtrates of an unidentified species of *Streptomyces*. These antibiotics are of special interest because of their high potency and their extreme toxicity. Because they are yellow in color and are produced by a *Streptomyces*, they have been named xanthomycin A and xanthomycin B.

The organism, designated *Streptomyces* 94, is one of a series isolated from oats, flax, and wheat. Because *Streptomyces* 94 had shown marked antagonistic activity against both Gram-positive and Gram-negative bacteria in a preliminary agar streak test, it was chosen for further study.

EXPERIMENTAL

Antibiotic Production by Streptomyces 94

Method of Assay—In the preliminary work on xanthomycin, a serial dilution type of assay was used in which *Staphylococcus aureus* H was the test organism. The *S. aureus* H dilution unit was adopted as the standard unit for xanthomycin. It is defined as that amount of antibiotic material which, when dissolved in 1 ml. of 0.75 per cent peptone and 0.25 per cent yeast extract medium at pH 7.2, will just inhibit the growth of *S. aureus* H during an incubation period of 20 hours at 37° when the inoculum per 10 ml. of medium is 1 drop of a 1:10 dilution of a 24 hour culture grown on the same medium.

The dilution assay was very laborious and inconvenient. It was useful for the preliminary work on xanthomycin, but a more convenient turbidimetric assay was developed for routine use. The medium for the turbidimetric assay was peptone 0.5 per cent, beef extract 0.3 per cent, and yeast extract 0.3 per cent in distilled water, adjusted to pH 7.6 to 7.7 with NaOH. 4 ml. of a culture of *Staphylococcus aureus* H grown on the same medium for 16 to 20 hours at 37° were used to inoculate each 100 ml. of assay medium.

A stock solution of partially purified xanthomycin was assayed against *Staphylococcus aureus* H by the dilution assay method and was found to con-

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This investigation has been aided by grants from The Upjohn Company, Kalamazoo, Michigan, and the Abbott Laboratories, North Chicago, Illinois.

† Junior Research Fellow, National Institute of Health.

tain 10,000 units per ml. This solution was stored in the refrigerator and its potency was checked frequently by the dilution assay method. For the turbidimetric assay this solution was diluted with sterile distilled water to contain 100 units per ml. For the standard curve 0.10, 0.08, 0.06, 0.04, and 0.02 ml. of a standard solution containing 100 units per ml. were pipetted in duplicate into a series of 18 × 150 mm. test-tubes. Similar quantities of a suitable dilution of the sample to be tested were pipetted into another series of tubes. 10 ml. of inoculated broth were pipetted into each tube with an automatic pipetting machine, and the tubes were then incubated in a water bath at 37° for 3 hours. At the end of the incubation period, growth was stopped by steaming the tubes for 10 minutes, and turbidities were read on a Lumetron model 402E colorimeter equipped with a 6500 A broad band filter. A standard curve, obtained by plotting arbitrary galvanometer readings against units of the antibiotic per tube, is shown in Fig. 1. Units per tube of the unknown sample were obtained from the standard curve. When several levels of an unknown sample fell within the range of the standard curve, the calculated potencies usually agreed within 5 per cent, and when a sample was assayed on successive days the results usually did not vary more than 5 to 7 per cent.

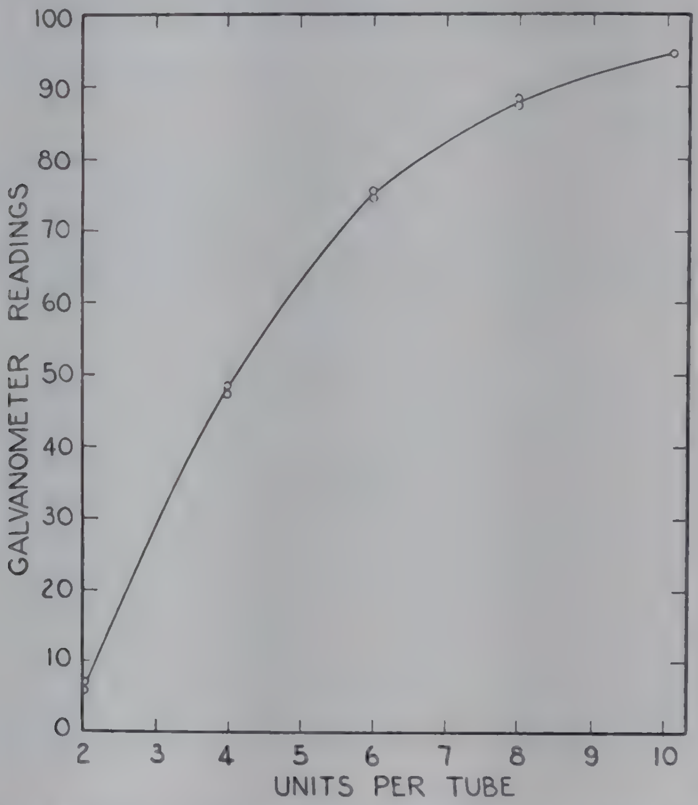


FIG. 1. Standard curve for xanthomycin turbidimetric assay

Fermentations in Shaken Flasks—The shaken flask technique was employed in experiments designed to find a good medium for antibiotic production. Cotton-plugged 500 ml. Erlenmeyer flasks containing 100 ml. of medium were autoclaved 30 to 40 minutes at 15 pounds of steam pressure and the cooled flasks were inoculated with a suspension of *Streptomyces* 94 spores grown on 6 ounce bottle plates of the following medium: glucose 1 per cent, asparagine 0.05 per cent, K_2HPO_4 0.05 per cent, agar 1.5 per cent, and distilled water. 20 ml. of spore suspension were prepared from each bottle plate and 2 to 5 ml. were used to inoculate each flask of medium. The inoculated flasks were incubated at 25° on a reciprocating shaker (4 inch strokes, 90 to 100 per minute), and samples were taken periodically for assay.

Approximately twenty-five different media were tested. The best of those tried consisted of 1 per cent each of corn steep solids, soy bean meal, dextrin (No. 151 of Corn Products Refining Company), K_2HPO_4, and NaCl, and 0.1 per cent of $CaCO_3$ in tap water. Yields on this medium were 5000 to 8000 units per ml. with the original culture. Maximum yields occurred in 4 to 5 days, after which the antibiotic concentration decreased rapidly if the fermentation was allowed to continue. When corn steep solids were omitted from the medium, good growth was obtained, but no antibiotic was produced. Peptone could be substituted for soy bean meal with good results, and lactose and glycerol were about as effective as dextrin.

Isolation of Better Producing Strains of Streptomyces 94—*Streptomyces* 94 is susceptible to its own antibiotic. Therefore an attempt was made to obtain more resistant strains and also better producers of the antibiotic.

Spores of strain 94 were plated out in glucose-asparagine agar containing 6000 units of partially purified antibiotic per ml. Control plates that contained no antibiotics were included. The plates were incubated at 30° for 2 to 3 days after which time colonies were isolated. Spore germination was apparently inhibited by the antibiotic, since many more colonies appeared on the control plates than on the plates containing the antibiotic.

The new strains were tested for antibiotic production in shaken flasks and the results are shown in Table I. Strain S-26 which was isolated from a control plate, did not produce any antibiotic. The other strains were all isolated from plates containing the antibiotic and gave yields that were 3 to 4 times higher than those produced by the original culture.

Fermentations in 30 Liter Fermenters—The 30 liter fermenters described by Peterson (1) were used for producing xanthomycin for purification studies. 12 liters of medium were placed in a fermenter, 75 to 100 ml. of a 2 per cent solution of octadecanol in lard oil were added as an antifoam, and the fermenter was autoclaved at 15 pounds of steam pressure for 105 minutes. After cooling, each fermenter was inoculated with 500 to 700 ml. of vegetative inoculum of strain 94 which had been grown 3 days on a shaker at 25°. Aeration was at the rate of 0.28 cubic feet per minute and the fermenters were stirred at the rate of 400 R.P.M.

Some of the chemical changes occurring in 30 liter fermenters were studied. The medium was the same as that used in shaken flasks except that 0.5 per cent of lactose was substituted for dextrin. Two of the new strains, Nos. S-33 and V-28, were compared with the original culture. They produced about 2.5 times as much of the antibiotic as the original strain. The chemical changes brought about by the three strains were very similar. The data for strain S-33 are presented in graphic form in Fig. 2.

TABLE I

Xanthomycin Production by New Strains of Streptomyces 94 in Shaken Flasks

Medium, corn steep solids 1 per cent, soy bean meal 1 per cent, K_2HPO_4 1 per cent, NaCl 1 per cent, lactose 0.5 per cent, $CaCO_3$ 0.1 per cent, and tap water.

Strain	3 days		3.5 days		4 days		4.5 days		5 days		5.5 days	
	pH	Units per ml.	pH	Units per ml.	pH	Units per ml.	pH	Units per ml.	pH	Units per ml.	pH	Units per ml.
Original	7.3	0	7.5	1,380	7.4	2,450	7.4	4960	7.8	5530	8.0	<5000
	7.3		7.5	1,640	7.4	2,900	7.5	4820	7.8	5260	8.0	<5000
94-S-26	7.1	0	7.4	0	7.6	0	7.8	0				
	7.1		7.4	0	7.5	0	7.8	0				
94-S-28	7.1	4550	7.4	>10,000	7.7	12,300	7.9	4200				
	7.1		7.3	>10,000	7.6	14,900	7.9	6500				
94-V-28	7.2	>5000	7.3	>10,000	7.5	20,400	7.8	7900				
	7.2		7.4	>10,000	7.5	20,400	7.8	8400				
94-S-29	7.1	>5000	7.4	>10,000	7.6	14,900	7.9	5300				
	7.1		7.4	>10,000	7.6	12,200	7.9	4600				
94-S-33	7.1	4100	7.3	>10,000	7.5	18,200	7.8	6300				
	7.1		7.3	>10,000	7.5	18,300	7.9	6100				
94-V-33	7.1	1145	7.4	7,460	7.4	14,400	7.7	9300				
	7.2		7.2	7,800	7.4	15,700	7.7	8800				
94-V-34	7.1	>5000	7.3	>10,000	7.6	17,100	7.8	6800				
	7.1		7.3	>10,000	7.6	15,400	7.8	6000				

The antibiotic began to appear early in the fermentation and reached a maximum around 40 hours, after which it decreased rapidly. The pH increased early in the fermentation, dropped as the yield of the antibiotic approached its maximum, and then rose slightly throughout the rest of the fermentation. Sugar, calculated as lactose, declined throughout the fermentation. Total soluble nitrogen decreased from the time of inoculation to the time of maximum yield of the antibiotic; it then began to increase, apparently owing to autolysis. There was very little ammonia nitrogen in the medium at the time of inoculation and it disappeared in the first 50 hours.

Analytical Methods—Kjeldahl nitrogen was determined by the micromethod of Johnson (2) and ammonia nitrogen by the method of Gailey *et al.* (3), except that the ammonia was removed by steam distillation rather than by aeration. For lactose the samples were autoclaved at 120° for 15 minutes in 0.75 N HCl, and reducing sugars were determined by the method of Shaffer and Somogyi (4). Reagent 50 with 5 gm. of KI per liter was used and the samples were heated in a boiling water bath for 25 minutes.

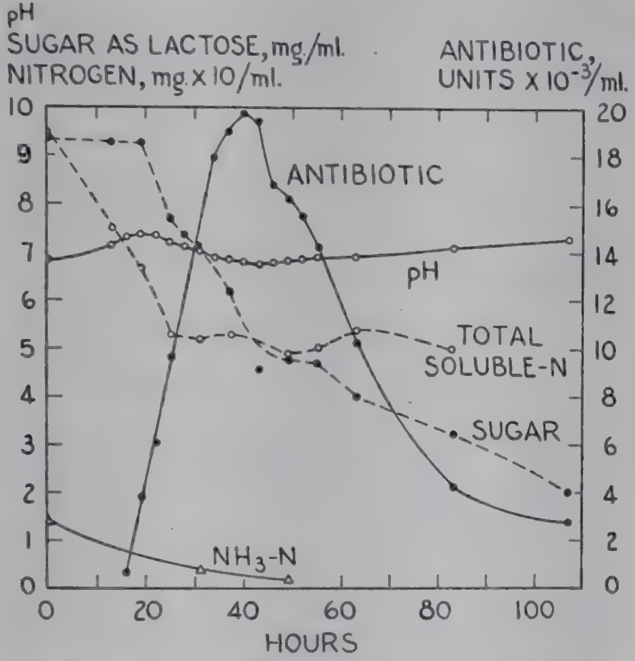


FIG. 2. Chemical changes in a *Streptomyces* 94 fermentation

Purification of Xanthomycin

Two major procedures were employed in concentrating xanthomycin. The active material in culture filtrates was first adsorbed on norit, from which it was eluted with 0.1 N HCl saturated with butanol. After concentration of the eluate, the antibiotic was extracted into chloroform and then into acidified water. It could be precipitated from concentrated water extracts with picric acid or ammonium reineckate.

Adsorption of Antibiotic on Norit and Elution—The mycelium was removed by filtration and after the pH of the culture filtrate was adjusted to 7.5 to 8.0 with NaOH, 2 to 3 per cent of norit was added. Usually approximately 1 per cent of Celite 545 was also added to aid in filtration. Small batches were stirred for approximately 1 hour and were then filtered through a Büchner funnel. Large batches were filtered through a filter press and the filtrate was pumped through three additional times to insure good adsorption. Generally 75 to 95 per cent of the active material was adsorbed on the norit.

The norit was washed with a volume of water saturated with butanol equal to one-third the volume of original culture filtrate. This removed much inactive material and only little of the antibiotic. The operation was conveniently carried out in the filter press by merely pumping the solution through after the norit had been collected in the press.

The antibiotic was eluted from the norit with 0.1 N HCl saturated with butanol. This was accomplished with small batches by suspending the norit in the eluent and stirring for 30 minutes. For large batches the eluent was usually divided into two portions and each portion was pumped twice through the norit in the filter press. Usually 80 to 90 per cent of the antibiotic was eluted with a volume of eluent equal to one-half that of the original culture filtrate. This adsorption and elution procedure resulted in a 3- to 5-fold purification based on units per mg. of dry weight. Data for adsorption and elution of both a small and a large batch are summarized in Table II.

TABLE II

Concentration of Xanthomycin by Norit Adsorption and Elution

	Experiment No.	Volume	Potency			Adsorbed	
		l.	*units per ml.*	*units per mg. (dry weight)*	*total units*	*units*	*per cent*
Adsorption, culture filtrate	1	11.9	8,155	315	97,073,000	90,528,000	93
	2	33.0	5,480	180	180,840,000	142,890,000	79
						Recovery from norit	Purification (dry weight basis)
						per cent	
Elution,* norit eluate	1	5.0	16,090	945	80,450,000	89	3-fold
	2	20.0	6,310	980	126,200,000	88	5.4-fold

* The eluent was 0.1 N HCl saturated with butanol. The procedure for Experiment 1 was carried out with the usual laboratory apparatus, while a filter press was used for adsorbing and eluting in Experiment 2.

Extraction with Chloroform—The amount of the antibiotic extractable from aqueous solution with solvents increased as the pH was raised from 2 to 8. Chloroform and butanol were the best solvents of those tried; less effective solvents were ethyl acetate, amyl acetate, and ether.

The eluate containing the active material was concentrated by distillation *in vacuo* to a small volume (500 to 2000 ml., depending on the size of the batch and the solids content). The pH was adjusted to 7.0 to 7.5 with NaOH, precipitated material was removed by centrifugation, and the concentrate was extracted with 2 to 3 equal volumes of chloroform. From the combined chloroform extracts, the active material was extracted into a small volume of water acidified to pH 2 or lower.

This extraction process brought about a 40- to 60-fold increase in purity of the antibiotic. If the water-chloroform-water extraction process was repeated, the purity of the antibiotic was usually increased a few fold. Data from a typical experiment are shown in Table III.

Precipitation of Active Material with Picric Acid and Ammonium Reineckate—After the water extract had been concentrated to a small volume (50 ml. or less) by distillation *in vacuo*, a saturated solution of picric acid in alcohol or ammonium reineckate in water was added drop by drop until a precipitate no longer formed. The use of either precipitant resulted in a 5- to 6-fold increase in purity. The purest reineckate obtained from aqueous concentrates prepared by the above extraction procedure had a potency of 264,000 units per mg., and the purest picrate had a potency of 293,500 units per mg. (Table III). Attempts to crystallize the derivatives which were of this order of potency were unsuccessful. However, it was possible to obtain a crystalline reineckate after the aqueous concentrate had been purified further by counter-current distribution as described below.

TABLE III

Concentration of Xanthomycin by Extraction with Chloroform and Precipitation with Picric Acid

Fraction	Quantity	Potency			Recovery	Purification (dry weight basis)
	ml.	*units per ml.*	*units per mg.*	*total units*	*per cent*	
1. Norit eluate	3010	20,170	980	60,711,700		
2. Water-chloroform-water concentrate	212	268,500	42,620	58,816,000	97	44-fold
3. Treatment in Fraction 2 repeated	225	275,500	58,620	61,875,500	102	60-fold
	mg.					
4. Picrate prepared from Fraction 3	163.2		293,500	47,899,200	79	300-fold

Purification by Counter-Current Distribution—The Craig (5, 6) technique of counter-current distribution was employed in purifying the active material further and separating its various components. As a preliminary experiment, the distribution of the antibiotic (hydrochloride) between chloroform and buffer at various levels of pH was determined. The distribution coefficients are given in Table IV. At pH 2.0 the antibiotic was practically all retained in the buffer phase, while at pH 8.0 over 90 per cent was extracted into the chloroform.

The distribution pattern obtained for an aqueous concentrate of the hydrochloride of the antibiotic in the Craig apparatus is shown in Fig. 3. Chloroform and 0.1 M phosphate buffer at pH 4.40 were the two phases. The lower portion of each tube in the Craig apparatus was filled with chloroform saturated with the buffer, and an equal volume, 8 ml., of buffer saturated with chloroform was added to the upper portions of Tubes 1 to 24. To the upper portion of Tube 0 were added 8 ml. (500,000 units) of a solution of the antibiotic in buffer saturated with chloroform. The apparatus was inverted thirty times and after the layers had separated the upper part of the apparatus was rotated one stage. This was repeated for twenty-four transfers. After the twenty-fourth transfer the buffer layer of each tube was acidified to pH 1.0 with concentrated HCl and the apparatus was again inverted a few times to extract the antibiotic into the buffer phase. Samples of the buffer layer were then withdrawn for assay.

TABLE IV

Distribution of Xanthomycin between Equal Volumes of Chloroform and 0.02 M Phosphate-Citrate Buffer*

pH	Distribution coefficient, $\frac{\text{chloroform}}{\text{buffer}}$
2.0	0.02
3.0	0.2
4.0	0.6
5.1	3.0
6.0	6.2
7.2	8.1
8.0	11.2

* The material used in this experiment was a mixture of approximately 90 per cent of xanthomycin A, 10 per cent of xanthomycin B, and 0.1 per cent of the third component as shown later by counter-current distribution studies.

The pattern (Fig. 3) shows that the material consisted of at least three antibiotic components. Similar patterns were always obtained for antibiotic concentrates. The main component, represented by the second peak, was named xanthomycin A, and it made up 65 to 90 per cent of the total antibiotic. The component represented by the first peak was named xanthomycin B and it ranged from 10 to 35 per cent of the total. The material which occurred in Tubes 23 and 24 never amounted to more than 1 per cent of the total. It was not named and no further work was done on it.

An antibiotic concentrate containing 290 million units in 60 ml. of aqueous solution was run through the Craig apparatus, 8 ml. at a time. The volume of the concentrate was not reduced further because the use of more concentrated solutions resulted in difficulties with emulsions. Chloroform and 0.6 M phosphate buffer at pH 4.40 were the two phases. After each 8 ml. portion was distributed in the apparatus, the contents of each tube were withdrawn and separated. The antibiotic from each chloroform portion was then extracted into an equal volume of 0.05 M phosphoric acid. All the fractions from the chloroform layer and all those from the buffer layer were kept separate. Corresponding fractions from each of the distributions were combined for assays and nitrogen determinations. The results are shown in Table V. Approximately 15 per cent of the total antibiotic material was present in the first peak, Tubes 0 to 6, approximately 85 per cent was present in the second peak, Tubes 7 to 23, and only 0.5 per cent was represented by the third component in Tube 24. The nitrogen figures show that nitrogen-containing impurities did not follow the same distribution pattern as the antibiotic. The nitrogen figures are not absolute values but are only relative because not all the nitrogen in the antibiotic appeared as Kjeldahl nitrogen.

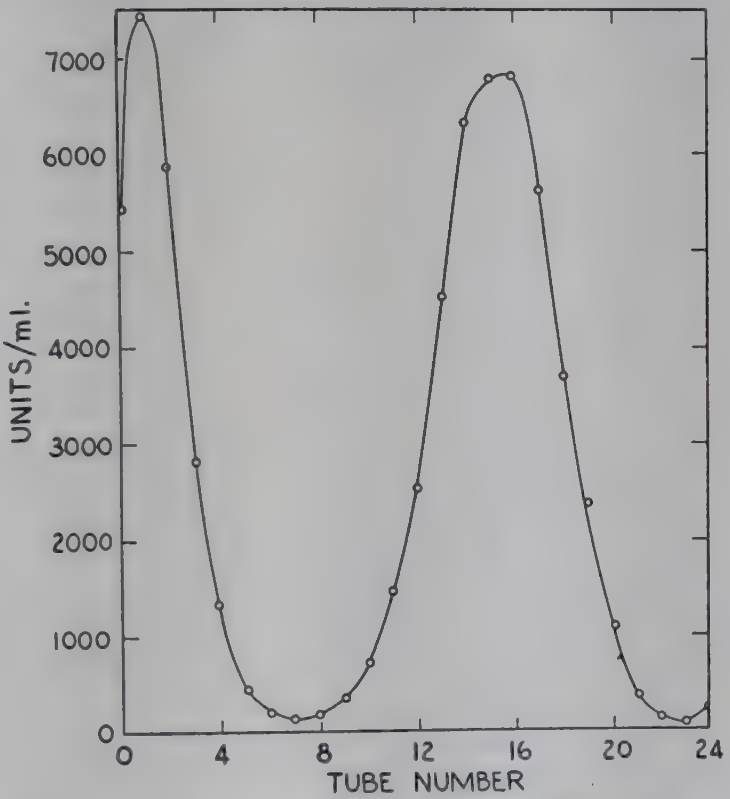


Fig. 3. Distribution pattern for a xanthomycin concentrate (chloroform-0.1 M phosphate system, pH 4.40).

Preparation of Crystalline Reineckate of Xanthomycin A—The fractions from the chloroform layers of Tubes 10 to 19 (Table V) were combined for preparation of the reineckate of xanthomycin A. A sample of the combined fractions was run through the Craig apparatus to test for antibiotic homogeneity. Chloroform and 0.3 M phosphate buffer at pH 4.25 were the two phases. The distribution pattern is shown in Fig. 4; there was only approximately 0.2 per cent of xanthomycin B present in the material.

TABLE V

*Craig Counter-Current Distribution Studies on Xanthomycin**

Tube No.	Chloroform layer			Buffer layer		
	Nitrogen	Antibiotic		Nitrogen	Antibiotic	
	γ per ml.	*units per ml.*	*units per γ N*	*γ per ml.*	*units per ml.*	*units per γ N*
0		6,800			229,000	
1		10,300			233,000	
2		13,500			122,000	
3		17,100			60,000	
4		18,900			33,000	
5		34,900			22,000	
6		41,800			24,000	
7		54,000			29,000	
8	10.6	70,100	6,610		39,000	
9		95,900			45,400	
10	13.7	122,000	8,910	15.0	70,000	4650
11		158,000		14.5	91,000	6250
12	23.0	217,000	9,430	17.3	126,000	7300
13		261,000		20.2	162,500	8050
14	31.0	336,000	10,800	25.5	200,000	7850
15		409,000		31.8	245,000	7700
16	32.4	410,000	12,600	24.8	222,000	8950
17		371,000		25.1	197,000	7850
18	25.7	281,000	10,900	17.7	137,000	7750
19		156,000		11.8	76,000	6450
20	17.2	88,000	5,120		38,000	
21	14.0	33,400	2,390		16,000	
22		11,100			4,800	
23		9,800			2,000	
24		21,800			2,400	

Original sample, 4720 units per microgram of N.

* The two phases were chloroform and 0.6 M phosphate buffer, pH 4.40.

The remainder of the combined fractions was used to prepare the reineckate as follows: The yellow aqueous solution was concentrated to 50 ml. and a saturated solution of ammonium reineckate in water was added drop by drop until a precipitate no longer formed. The precipitate which was partially crystalline was collected and dried. It weighed 170 mg. and had a potency of 383,000 units per mg. The reineckate was recrystallized by dissolving it in a minimum amount of 95 per cent ethanol at 50° and allowing the solution to cool slowly. The recrystallized product was in the form of needles and its potency was 460,000 units per mg. After a second recrystallization the potency had increased to 490,000 units per mg. and it was not changed by a third recrystallization. The final yield was 70 mg. of long orange needles, m.p. 165–170° (decomposition). It was quite insoluble in cold water, soluble in warm methanol, and very soluble in acetone.

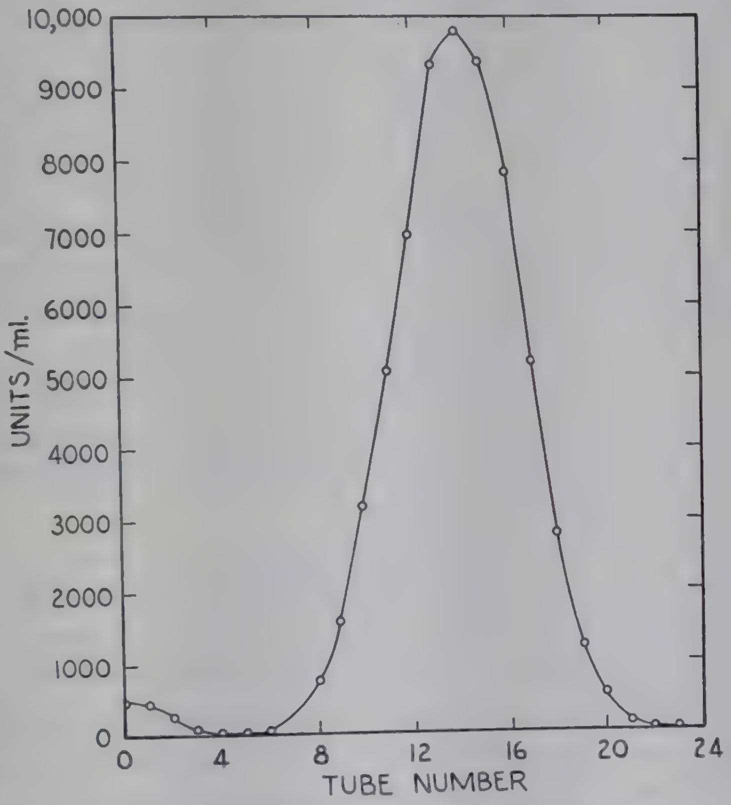


FIG. 4. Distribution pattern for xanthomycin A hydrochloride (chloroform-0.3 M phosphate system, pH 4.25).

Chemical analysis (Clark Microanalytical Laboratory) gave the following figures:

$C_{38}H_{57}N_{12}O_{13}S_4Cr$.	Calculated.	C 42.64, H 5.37, N 15.70, S 11.98
	Found.	" 42.68, " 5.40, " 15.43, " 12.10

The calculated equivalent weight of the antibiotic based on the sulfur content of the Reinecke ion, which made up 30 per cent of the total weight of the reineckate, is 742. Results of diffusion rate determinations on xanthomycin A hydrochloride by the method of McBain and Liu (7) indicate that the molecular weight is the same as the equivalent weight.

Another reineckate preparation of xanthomycin A was made in the same manner from the buffer fractions of Tubes 11 to 19 (Table V). After two recrystallizations from ethanol it had a potency of 490,000 units per mg. and was apparently identical with the first preparation.

Regeneration of Xanthomycin A Hydrochloride from Crystalline Reineckate—A 20.2 mg. sample of crystalline reineckate of xanthomycin A was dissolved in 1 ml. of methanol warmed to 50°. A drop of concentrated HCl was added and the hydrochloride was precipitated by pouring 25 ml. of anhydrous ether into the methanol solution. The precipitate was removed by centrifugation and was dissolved in a few drops of anhydrous methanol, from which it was again precipitated by the addition of anhydrous ether. It was then filtered, washed with anhydrous ether, and dried in a vacuum desiccator. 13 mg. of the hydrochloride with a potency of 565,000 units per mg. were thus obtained. The calculated potency of the hydrochloride, based on the potency and composition of the reineckate, is approximately 670,000 units per mg. The discrepancy between the calculated and observed potencies cannot be entirely explained. Although the discrepancy may be attributed in part to assay error, it suggests that there was a loss in activity upon regenerating the hydrochloride from the reineckate.

Xanthomycin A hydrochloride was a yellow amorphous material very soluble in water and methanol. It gave negative Sakaguchi, Molisch, ninhydrin, and ferric chloride tests. The nitroprusside test for sulfur on a sample fused with sodium was negative.

An absorption curve for the hydrochloride of xanthomycin A, read on a Beckman quartz spectrophotometer, is shown in Fig. 5. The curve shows two maxima, with the first at 264 to 267 mμ and the second at 325 to 327 mμ.

Stability of Xanthomycin—The results of stability tests on xanthomycin are shown in Table VI. The antibiotic was stable in acid solution and unstable at pH 6.0. There was no loss of activity when the antibiotic stood in 3 N HCl at room temperature for 1 hour. When the solutions were heated in a boiling water bath for 25 minutes, there was a 90 per cent loss of activity at pH 6.0 and only 14 per cent loss at pH 1.0.

Comparison of Xanthomycins A and B—A crystalline derivative of xanthomycin B was not obtained. The most pure preparations were yellow and had solubility properties similar to those of xanthomycin A. Picrates of xanthomycins A and B had similar bacterial spectra and were of equal toxicity to mice.

Bacterial Spectrum of Xanthomycin—A serial dilution type of assay was used in determining the bacterial spectrum of xanthomycin. The medium was 0.75 per cent peptone and 0.25 per cent yeast extract in distilled water, adjusted to pH 7.2 with NaOH. The test organisms were grown on the same medium for 24 hours and 1 drop of a 1:10 dilution of the cultures was

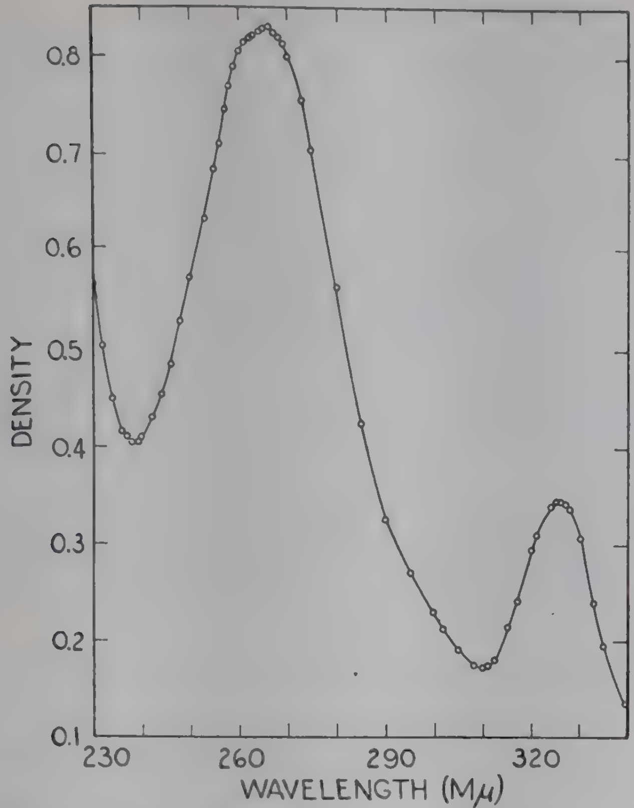


FIG. 5. Absorption spectrum of xanthomycin A hydrochloride (concentration, 4.5 mg. per 100 ml. of water).

TABLE VI

*Stability of Xanthomycin**

Treatment	Activity lost
	per cent
pH 6.0, boiling water bath, 25 min.	90
" 2.0, " " " 25 "	30
" 1.0, " " " 25 "	14
1 N HCl, " " " 25 "	23
2 " " " " " 25 "	27
3 " " " " " 25 "	45
1 " " room temperature, 60 "	0
2 " " " " 60 "	0
3 " " " " 60 "	0

* The material tested was a mixture of approximately 90 per cent of xanthomycin A, 10 per cent of xanthomycin B, and 0.1 per cent of the third component.

used to inoculate each assay tube containing 9 to 10 ml. of the medium. After the tubes had been incubated for 16 to 20 hours, the highest dilution of the sample causing complete inhibition of growth as estimated by visual inspection was recorded.

TABLE VII

Antibacterial Activity of Xanthomycin A Hydrochloride and Actinomycin

Organism	Highest dilution of inhibition*	
	Actinomycin	Xanthomycin A hydrochloride
S. aureus H	$1:2.9 \times 10^7$ $(1:4.3 \times 10^7)$	$1:5.75 \times 10^8$ $(1:6.05 \times 10^8)$
B. subtilis II	$1:2.9 \times 10^7$ $(1:4.3 \times 10^7)$	$1:1.53 \times 10^8$ $(1:3.0 \times 10^8)$
E. coli H-52	$1:3.0 \times 10^4$ $(1:6.0 \times 10^4)$	$1:3.0 \times 10^6$ $(1:6.0 \times 10^6)$
S. marcescens	$1:3.0 \times 10^4$ $(1:6.0 \times 10^4)$	$1:6.0 \times 10^6$ $(1:9.0 \times 10^6)$

* The figures in parentheses are the next highest dilutions at which growth occurred.

TABLE VIII

Bacterial Spectrum of Picrate of Xanthomycin (293,500 Units per Mg.)

Organism	Highest dilution of inhibition*
Staphylococcus aureus H	$1 : 3.0 \times 10^8$ $(1 : 4.0 \times 10^8)$
“ *albus*	$1 : 2.4 \times 10^9$ $(1 : 3.2 \times 10^9)$
Micrococcus subcitreus	$1 : 2.4 \times 10^8$ $(1 : 2.8 \times 10^8)$
“ *flavescens*	$1 : 2.0 \times 10^8$ $(1 : 2.4 \times 10^8)$
Escherichia coli H-52	$1 : 2.4 \times 10^6$ $(1 : 4.0 \times 10^6)$
Proteus vulgaris	$1 : 2.4 \times 10^6$ $(1 : 2.8 \times 10^6)$
Serratia marcescens	$1 : 4.0 \times 10^6$ $(1 : 8.0 \times 10^6)$
Aerobacter aerogenes	$1 : 4.0 \times 10^6$ $(1 : 4.0 \times 10^7)$
Salmonella gallinarum	$1 : 2.4 \times 10^6$ $(1 : 4.0 \times 10^6)$
Alcaligenes viscosus	$1 : 4.0 \times 10^7$ $(1 : 8.0 \times 10^7)$
Sarcina lutea	$1 : 4.0 \times 10^7$ $(1 : 6.0 \times 10^7)$
Bacillus subtilis I	$1 : 2.4 \times 10^8$ $(1 : 2.8 \times 10^8)$
“ *mycoides*	$1 : 2.8 \times 10^7$ $(1 : 3.2 \times 10^7)$
“ *mesentericus*	$1 : 4.0 \times 10^6$ $(1 : 8.0 \times 10^6)$
“ *megatherium*	$1 : 4.0 \times 10^6$ $(1 : 4.0 \times 10^7)$
“ *cereus*	$1 : 2.4 \times 10^6$ $(1 : 2.8 \times 10^6)$
“ *niger*	$1 : 2.0 \times 10^8$ $(1 : 2.5 \times 10^8)$
“ *brevis*	$1 : 4.0 \times 10^6$ $(1 : 4.0 \times 10^7)$
“ *albolactis*	$1 : 4.0 \times 10^5$ $(1 : 4.0 \times 10^6)$
“ *graveolens*	$1 : 4.0 \times 10^5$ $(1 : 4.0 \times 10^6)$
“ *fusiformis*	$1 : 8.0 \times 10^7$ $(1 : 1.2 \times 10^8)$
“ *vulgatus*	$1 : 2.0 \times 10^7$ $(1 : 4.0 \times 10^7)$
“ *subniger*	$1 : 4.0 \times 10^7$ $(1 : 8.0 \times 10^7)$
Mycobacterium tuberculosis (avian)	1 : 2000
“ “ (BCG)	1 : 2000
“ “ (TB-1)	1 : 2000

* The figures in parentheses are the next highest dilutions at which growth occurred.

The hydrochloride of xanthomycin A, regenerated from the crystalline reineckate, and crystalline actinomycin were compared as to their activities against four organisms. The results are shown in Table VII. Against *Staphylococcus aureus* H xanthomycin A was 19 times as active as actinomycin and against *Escherichia coli* it was 100 times as active.

A picrate which was prepared from some antibiotic material that had not been separated into its components and which had a potency of 293,500 units per mg. was tested against twenty-six organisms. The results are shown in Table VIII. The organisms included seventeen species of Gram-positive bacilli and micrococci, six Gram-negative species, and three strains of *Mycobacterium tuberculosis*. The antibiotic was particularly active against the micrococci; the effective dilutions for inhibiting growth ranged from $1:2.0 \times 10^8$ for *Micrococcus flavescens* to $1: 2.4 \times 10^9$ for *Staphylococcus albus*. Gram-positive bacilli were inhibited at dilutions ranging from $1:4.0 \times 10^5$ for *Bacillus graveolens* to $1:2.4 \times 10^8$ for *Bacillus subtilis* I. The material was effective against Gram-negative organisms at dilutions ranging from $1 : 2.4 \times 10^6$ for *Escherichia coli* to $1 : 4.0 \times 10^6$ for *Serratia marcescens*. The strains of *Mycobacterium tuberculosis* were not inhibited at dilutions as low as 1:2000.

TABLE IX

*Intravenous Toxicity of Xanthomycin A Hydrochloride to Mice**

No. of mice	Amount injected		Total No. dead at			No. of survivors
			24 hrs.	48 hrs.	72 hrs.	
	units	γ				
4	14,460	25.6	2	4	4	0
6	7,232	12.8	1	4	5	1
8	3,616	6.4	1	6	6	2
6	1,808	3.2	0	3	3	3
3	904	1.6	0	0	0	3

* Each of the mice weighed approximately 20 gm.

Toxicity of Xanthomycin to Mice—The results of mouse toxicity tests on xanthomycin A hydrochloride regenerated from the crystalline reineckate are shown in Table IX. As little as 3.2 γ or 1808 units per 20 gm. mouse was toxic. The purest preparation of xanthomycin B was equally toxic when doses were expressed on the basis of units of antibiotic.

Observed symptoms usually included labored breathing, lethargy, anorexia, and muscular spasms. Autopsies revealed normal organs with the exception of hemorrhagic lungs.

SUMMARY

Two new antibiotics have been obtained from culture filtrates of an unidentified species of *Streptomyces*. The two appear to be very similar

and because of their yellow color and source they were named xanthomycin A and xanthomycin B. Xanthomycin A was obtained in the form of a crystalline reineckate, but xanthomycin B did not yield a crystalline derivative. Both of the antibiotics are solvent-soluble basic compounds active against Gram-positive and Gram-negative organisms, and both are extremely toxic to mice. Xanthomycin A hydrochloride, regenerated from the crystalline reineckate, was toxic in amounts as low as 3.2 γ per 20 gm. mouse.

The authors are indebted to Professor Elizabeth McCoy and her research assistants, F. R. Hanson, who isolated the culture, and Barbara Sargeant, Mary A. Roberts, and A. P. Saunders, who determined the toxicity of xanthomycin to mice. They also wish to acknowledge the assistance of H. E. Arkens in some of the experimental work and to thank Dr. S. A. Waksman for a sample of actinomycin.

BIBLIOGRAPHY

1. Peterson, W. H., *Harvey Lectures*, **42**, 276 (1946–47).
2. Johnson, M. J., *J. Biol. Chem.*, **137**, 575 (1941).
3. Gailey, F. B., Stefaniak, J. J., Olson, B. H., and Johnson, M. J., *J. Bact.*, **52**, 129 (1946).
4. Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, **100**, 695 (1933).
5. Craig, L. C., *J. Biol. Chem.*, **155**, 519 (1944).
6. Craig, L. C., Golumbic, C., Mighton, H., and Titus, E., *J. Biol. Chem.*, **161**, 321 (1945).
7. McBain, J. W., and Liu, T. H., *J. Am. Chem. Soc.*, **53**, 59 (1931).

FURTHER OBSERVATIONS ON THE LIPOTROPIC NEED FOR INOSITOL

By M. L. MacFARLAND and E. W. McHENRY

(*From the Department of Public Health Nutrition, School of Hygiene, University of Toronto, Toronto, Canada*)

(Received for publication, July 15, 1948)

Inositol was originally shown to be a lipotropic agent in rats maintained under special conditions (1); the animals were depleted of fat and of B vitamins by maintenance for 3 weeks on a fat-free, high carbohydrate diet, and during a subsequent week fat synthesis was promoted by a supplement of B vitamins and a beef liver fraction. Under these conditions the rats developed markedly fatty livers containing large amounts of cholesterol. These fatty livers were produced despite the provision of choline, both in the liver fraction and as a supplement. The accumulation of fat and cholesterol in the liver was prevented by administration of inositol. The lipotropic action of inositol in rats has been confirmed by Engel (2), by Forbes (3), and by Handler (4), and has been demonstrated in humans by Abels *et al.* (5).

Several explanations of the production of fatty livers resistant to choline but responsive to inositol have been offered. In early work in this laboratory, crude preparations of biotin appeared to be equivalent to the beef liver fraction in producing the effect; the term "biotin fatty liver" was used to describe the fatty liver responsive to inositol. Subsequent investigation with pure biotin showed that this concept was erroneous (6); the biotin explanation has been fully criticized by Best *et al.* (7). In several studies in our laboratory, attempts were made to define further the dietary conditions necessary to cause fatty livers characterized by a high cholesterol content (resulting from synthesis) and by resistance to choline. By means of the same basal regimen, it was found that, when thiamine was the only supplement, the fatty livers were completely responsive to choline (8). With the addition of the other B vitamins increasing resistance to choline and responsiveness to inositol were observed (8); the full effect was secured only when beef liver fraction was furnished (6). Handler (4) concluded that the beef liver fraction could be replaced by biotin plus folic acid and has also stated that the effect was due to a stimulation of food consumption.

In this paper further efforts to explain the production of fatty livers showing resistance to choline and responsiveness to inositol are reported. While it was found that the beef liver fraction could be partially fractionated, further work along this line appeared to be unnecessary because of results obtained with pure substances.

Methods

The rats used were Wistar strain animals of both sexes reared in the colony of the Connaught Medical Research Laboratories. After attaining an average weight of 90 to 100 gm., they were provided with a fat-free, high carbohydrate diet containing no source of B vitamins (9). At the end of 3 weeks, having decreased in body weight by approximately one-third, the animals were divided into strictly comparable groups of generally ten rats, except in some cases when nine were used. During the subsequent week, various supplements were administered to different groups. Each rat was given the basal B vitamin supplement daily by subcutaneous injection and received the following amounts: thiamine hydrochloride 25 γ, riboflavin 25 γ, pyridoxine hydrochloride 40 γ, calcium pantothenate 100 γ, *p*-aminobenzoic acid 100 γ, and nicotinic acid 100 γ. The additional supplements under investigation were provided orally, mixed with the food, and the dosages were as follows: choline 20 mg., inositol 25 mg., folic acid 5 γ, biotin 5 γ. When abnormal quantities of B vitamins were given, the following amounts were provided in the food in addition to the injected basal amounts: thiamine 75 γ, riboflavin 430 γ, calcium pantothenate 2.4 mg., and nicotinic acid 6.5 mg.

The beef liver fraction, similar to that used previously (10), was obtained from the Connaught Medical Research Laboratories and the procedure of Hutchings *et al.* (11) was adopted in the preparation of norit eluate fractions from it. Crude liver fraction was diluted, adsorption on Pfanstiehl norit A was carried out at pH 3.0, and after washing the norit with 50 per cent ethanol, it was eluated twice at 70° with ammonia-ethanol solution. The combined eluates were finally concentrated *in vacuo* to the original volume of the liver fraction. The filtrate obtained after separation of the norit was neutralized and concentrated to appropriate volume. The daily dosage of these preparations was 2 cc. per rat.

At the end of the test week each animal received an intraperitoneal injection of nembutal, the livers were removed, and the crude fatty acids of the livers and carcasses were determined by methods described in previous publications (9, 12). In all cases, analyses were made on the pooled livers and pooled carcasses for each group. The total cholesterol content of the petroleum ether extracts was estimated by a modification of the procedure of Schoenheimer and Sperry (13). Data for carcass fats are not included in the reported results because they are not pertinent to the discussion. All data for liver lipides are results obtained by the analysis of tissues pooled for each group of animals. The figures presented have been selected as typical from a much larger series of similar observations.

Results

Typical effects on liver lipides caused by feeding various beef liver preparations in one of several experimental series are given in Table I. Attention is drawn to the characteristic effect of the liver fraction in causing livers high in fat and in cholesterol despite the supply of choline, both in the fraction and as an additional supplement. Throughout these studies, the criterion of potency has been the ability to produce fatty livers rich in cholesterol *when* choline was supplied. It is apparent that the eluate fraction exhibited effects very similar to those obtained when the original material was provided. The filtrate preparation contained some activity and it is obvious that adsorption had not caused complete fractionation. The activity of inositol, particularly on liver cholesterol, is clearly evident.

TABLE I

Effects of Beef Liver Fractions, Biotin, Folic Acid, and Abnormal Amounts of B Vitamins on Rat Liver Lipides

Supplements	Liver					
	Crude fatty acids			Cholesterol		
	mg.	*per cent*	*per cent of Group I**	*mg.*	*per cent*	*per cent of Group I**
Original liver fraction, choline (Group I)	2170	21.1	100	125	1.21	100
Original liver fraction, choline, inositol	456	5.8	21	25	0.33	20
Liver eluate, choline	2078	25.3	96	93	1.14	74
" filtrate, choline	1170	15.6	54	63	0.84	50
Folic acid, choline	635	11.1	29	26	0.46	21
" " biotin, choline	993	14.8	46	41	0.61	33
Additional B vitamins, choline	1303	18.3	60	49	0.70	39
" " " biotin, folic acid, choline	2185	25.7	101	94	1.10	75
Additional B vitamins, biotin, folic acid, choline, inositol	546	9.1	25	28	0.47	22

* Values in these columns are calculated by the equation,

$$\frac{\text{Average weight of liver fatty acids or cholesterol in particular group}}{\text{Average weight of liver fatty acids or cholesterol in Group I}} \times 100$$

Since Handler had reported (4) the joint efficacy of biotin and folic acid in simulating the action of the liver fraction, the effects of feeding this combination were examined. The results of a typical experiment in this series are reported in Table I.

It is clear that the administration of folic acid as sole replacement for liver fraction did not result in the development of fatty livers with the characteristics of the "liver fraction fatty liver." The lipide accumulation obtained when biotin and folic acid were both provided with choline did not contain as much fat nor as high a proportion of cholesterol as was obtained with the use of liver fractions. The present evidence does not substantiate Handler's conclusion (4) that the action of liver fraction in these experiments can be duplicated by administering biotin and folic acid without other additional measures.

Although the basal B vitamin supplement would be expected to be adequate under most conditions, it seemed reasonable to investigate the possibility that the additional quantities of vitamins provided by the liver fraction were related to the effects obtained. Consequently, the activity of further dietary supplements (equivalent to the vitamin content of the liver preparation and given in detail under "Methods") of thiamine, riboflavin, calcium pantothenate, and nicotinic acid were tried. The results, reported in Table I, show that feeding of additional amounts of these four factors failed to produce the same effects as the liver preparations, although the quantity of fatty acids was more similar than with other supplements. However, when biotin and folic acid, as well as additional thiamine, riboflavin, pantothenate, and nicotinic acid were provided, the liver lipide responses were very similar to those observed subsequent to liver supplementation. The results of one of five similar experiments are reported in Table I. Even with generous provision of choline, fatty livers containing a high proportion of cholesterol were obtained. The final line in Table I shows the lipotropic effect of inositol on the fatty liver produced by giving

Table II.

Effects of Restricted Food Intake on Liver Lipides

Group No.	Supplements* in addition to basal B vitamins	Weight gain in test week	Food consumption	Liver				
				Weight	Crude fatty acids		Cholesterol	
		gm.	*gm. per rat per day of test*	*gm.*	*mg.*	*per cent*	*mg.*	*per cent*
1	Biotin, folic acid, choline	30	10.8	7.1	1073	15.1	43.6	0.61
2	Biotin, folic acid, additional B vitamins, choline	26	10.2	7.6	1675	22.2	81.0	1.07
3	" "	38	12.1	9.5	2335	24.6	117.5	1.24

* The animals were fed *ad libitum* with the exception of the members of Group 2; they were permitted only as much food as the members of Group 1 on the corresponding day of the test.

biotin and folic acid in conjunction with abnormal amounts of other B vitamins.

Under the special conditions of our experiments, the animals exhibit a distinct need for dietary inositol for lipotropic purposes. Because Handler (4) has suggested that this augmented need for inositol is related to temporary stimulation of food consumption, an experiment was carried out in which the animals receiving biotin, folic acid, and the additional quantities of the other B vitamins were given only as much food as animals developing fatty livers which were much more responsive to choline; that is, animals without the augmented amounts of B vitamins. The results are described in Table II. A comparison of Groups 1 and 2, which had approximately the same amount of food, indicates that restriction of food consumption in Group 2 did not eliminate the effects of the additional vitamin factors on the liver lipides, but it is evident that food intake also had an effect in producing the high cholesterol fatty liver resistant to choline.

DISCUSSION

Under the conditions of our experiments, fatty livers are produced in a few days by the rapid synthesis of fat, presumably from carbohydrate. When thiamine is the only B vitamin supplement, the liver fat can be maintained at a normal level by supplying one lipotropic agent, choline (8). The addition of other B vitamins causes fatty livers which are not completely responsive to choline but which are amenable to choline and inositol. The most marked resistance to choline was observed previously (10) when a beef liver fraction was added to the vitamin supplements; this observation was made when pure biotin and folic acid were not available. The hypothesis that the liver fraction was active because of its biotin content was found to be untenable (5). The suggestion of Handler (4) that biotin and folic acid, given with customary amounts of other B vitamins, will simulate the liver fraction has not been confirmed by us. The question as to whether the activity of the liver fraction is due to an unidentified constituent or to its supply of extra amounts of a number of B vitamins appears to have been settled in favor of the latter explanation and further fractionation of the liver preparation seems unnecessary. All of our observations indicate that a fatty liver, at least of the type produced by *in vivo* fat synthesis, is made resistant to choline and responsive to inositol by increasing the intake of B vitamins, both in kind and in quantity.

No clear explanation is available as to why liver fat can be demobilized by choline alone under some circumstances and why inositol must be provided under other conditions. Presumably both substances are lipotropic because they promote the formation of phospholipides. We suggested (6) previously that different fatty acids may be involved; no further information on this point is available. In every experiment in this laboratory inositol has exerted a greater effect on liver cholesterol than has choline and we have observed instances in which inositol has markedly reduced liver cholesterol without any definite effect on fatty acids. Handler (4) has suggested that a large increase in food intake, with a surge in fatty acid synthesis, may be the factor causing choline resistance and inositol responsiveness. Pair feeding tests, of which a typical one is reported above, show that food consumption is a contributing factor but there is also a specific effect from the B vitamin supplements.

SUMMARY

Fatty livers occurring in choline-fed rats and susceptible to inositol have been produced in animals maintained on a fat-free, high-carbohydrate diet by supplying (*a*) crude beef liver fraction, (*b*) liver fraction eluate, or (*c*) biotin and folic acid with abnormal amounts of other B vitamins. Biotin and folic acid did not exhibit this effect unless the amounts of other B vitamins were abnormal. The action of the first two supplements is apparently explained by the third. The combined supplements have a specific effect which is augmented by an increased food consumption.

BIBLIOGRAPHY

1. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **139**, 485 (1941).
2. Engel, R. W., *J. Nutr.*, **24**, 175 (1942).
3. Forbes, J. C., *Proc. Soc. Exp. Biol. and Med.*, **54**, 89 (1943).
4. Handler, P., *J. Biol. Chem.*, **162**, 77 (1946).
5. Abels, J. C., Kupel, C. W., Pack, G. T., and Rhoads, C. P., *Proc. Soc. Exp. Biol. and Med.*, **54**, 157 (1943).
6. MacFarland, M. L., and McHenry, E. W., *J. Biol. Chem.*, **159**, 605 (1945).
7. Best, C. H., Lucas, C. C., Patterson, J. M., and Ridout, J. H., *Biochem. J.*, **40**, 368 (1946).
8. Gavin, G., Patterson, J. M., and McHenry, E. W., *J. Biol. Chem.*, **148**, 275 (1943).
9. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **132**, 41 (1940).
10. McHenry, E. W., and Gavin, G., *J. Biol. Chem.*, **134**, 683 (1940).
11. Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, **141**, 521 (1941).
12. McHenry, E. W., and Gavin, G., *J. Biol. Chem.*, **125**, 653 (1938).
13. Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, **106**, 745 (1934).

AN IMPROVED DITHIZONE METHOD FOR THE DETERMINATION OF SMALL QUANTITIES OF ZINC IN BLOOD AND TISSUE SAMPLES*

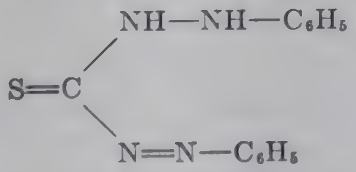
By BERT L. VALLEE and JOHN G. GIBSON, 2nd

(From the Medical Clinic of the Peter Bent Brigham Hospital, the Department of Medicine, Harvard Medical School, Boston, and the Department of Physics, Massachusetts Institute of Technology, Cambridge)

(Received for publication, July 11, 1948)

The study of zinc metabolism in experimental animals and humans is dependent upon a reliable analytical method. Numerous methods, gravimetric, turbidimetric, and colorimetric (1), have been tried and found wanting. Fischer (2) first introduced diphenylthiocarbazone (dithizone) for the analysis of metals with relation to industrial processes. This organic dye combines with a number of the heavy metals (Sandell (3)). The combination is selective for any one metal, depending upon the pH of the solution containing the metals, and the presence of salts which form complexes with other metals in solution. It was not until 1937 that Fischer and Leopoldi (4) adapted the dithizone method to the analysis of inorganic zinc. Holland and Ritchie (5) and Cowling and Miller (6) used this dye for zinc analyses in plants, and Hove *et al.* (7) in the measurement of carbonic anhydrase. The procedure employed by these authors, however, involved a preliminary extraction of other metals, principally copper, and a final extraction of zinc. Gettler (8) simplified the method by the use of a buffered solution of complex-forming salts, obviating the preliminary separation. We have further refined the method to obtain greater accuracy in the analysis of the zinc content of samples of whole blood, plasma, erythrocytes, and leucocytes and samples of tissue. Samples of the size which can be practicably obtained may contain as little as 1 to 5 γ of zinc.

Diphenylthiocarbazone has the following structural formula:



The dye is soluble in chloroform and carbon tetrachloride and insoluble in water. It decomposes in aqueous alkaline solutions. When dissolved in chloroform or carbon tetrachloride, the dye is dark green in transmitted and Bordeaux red in reflected light. It does not fade on standing when stored in the dark at 4°. When exposed to sunlight the dye is oxidized, with the production of a yellow color.

---

* The studies reported herein were supported by a grant-in-aid from the National Institute of Health.

At pH 5.5 and in the presence of a tartrate solution and complex-forming buffer, dithizone combines with zinc in stoichiometric proportions to form zinc dithizonate, but does not combine with other metals which may be present. The completion of this reaction is accompanied by a change of color of the dithizone from green to bright red. In routine extraction of samples this color change occurs slowly and appears to pass through an intermediary purple stage. In our experience, this color change takes place more rapidly when the dye is dissolved in carbon tetrachloride than in chloroform.

In procedures previously described, an excess of dithizone was added to insure the combination of all the zinc with dye. The excess dithizone was removed from the extracted zinc dithizonate by washing with dilute ammonia. This step, however, may introduce a considerable error because it is extremely difficult to determine the end-point of the washing process. In the technique described, the excess dithizone is not removed. The amount of zinc dithizonate present is determined by colorimetry at two critical wave-lengths, as described below.

Reagents—All reagents must be absolutely zinc-free. The best grade of chemically pure reagents should be obtained and, in our experience, even these may contain zinc in amounts sufficient to result in significant errors in the range of zinc content in which we are interested. Reagents therefore may require purification, as described below.

1. Diphenylthiocarbazone (Eastman Kodak). 100 mg. are dissolved in 1000 cc. of carbon tetrachloride, C.P. This stock solution is diluted to 1 mg. per cent for the extraction of very small quantities of zinc (1 to 20 γ). This solution should be stored at 4–6° and protected from sunlight at all times. No observable change in the solution occurs on standing at room temperature for a few hours.

2. Buffer solution. 556 gm. of $Na_2S_2O_3$, C.P., 90 gm. of C_3COONa, C.P., and 10 gm. of KCN, C.P., are dissolved in 1000 cc. of zinc-free water. The solution is then titrated with 15 N CH_3COOH to an approximate pH of 5.5, with methyl red as an indicator. A final adjustment to pH 5.5 is then made with a sensitive pH meter. The solution is then made up to 2000 cc. with zinc-free water in a volumetric flask. The buffer is then shaken with dithizone in carbon tetrachloride to remove any contaminating zinc, the extraction being repeated in a 500 cc. separatory funnel until the dithizone remains a clear green.

3. Tartrate solution. A 20 per cent solution of $NaKC_4H_4O_6 \cdot 4H_2O$,

C.P., is made up with zinc-free water. The solution is extracted with dithizone as in (2).

4. 0.1 N NH_4OH, C.P.
5. Concentrated NH_4OH, C.P.
6. Methyl red indicator, 1:100 alcoholic solution.

Cleaning of Glassware—Pyrex glassware must be used throughout the procedure since ordinary soft glass contains zinc. Particular precautions must be observed to prevent contamination with zinc. *All* glassware, including separatory funnels, transfer funnels, volumetric flasks, beakers, transfer and capillary pipettes, colorimetric tubes, test-tubes, and syringes for drawing blood, is washed with soap and water, rinsed with single distilled H_2O, and then immersed in a bath of 2 N HNO_3 for a minimum of 6 hours, preferably overnight. On removal from the acid, the glassware is rinsed with zinc-free water, to which a few drops of methyl red have been added, until disappearance of the indicator's red color demonstrates the removal of all acid. Satisfactory zinc-free water may be obtained by double distillation, the second of which is done in an all-Pyrex glass still. Previous to use, separatory funnels are then shaken (several times if necessary) with about 20 cc. of buffer solution and about 5 cc. of 1 mg. per cent dithizone solution, until the dithizone in the funnel remains green. The dithizone is discarded. An identical procedure is employed for cleaning volumetric flasks. In addition, flasks are then rinsed with 0.01 N NH_4OH until yellow to methyl red. This insures that the pH of any residual rinsing water will be within the range of alkalinity at which the extracted zinc dithizonate is not affected.

For routine dry ashing of blood samples which contain only a total of from 1 to 20 γ of zinc, platinum crucibles must be used. In this range porcelain, Vicor, Pyrex, and quartz crucibles have been found unsatisfactory, since apparently all contain minute quantities of zinc. They may be used safely for tissue samples, provided the aliquots are large enough so that the error introduced is negligible. Both platinum and porcelain crucibles are cleaned by boiling 2 N HCl in the vessel for at least 30 minutes, after which they are rinsed several times with zinc-free water.

Method

We have routinely analyzed whole blood, erythrocyte, leucocyte, and plasma fractions thereof (9), and samples of internal organs, bone, and urine. The material is placed in the crucible and slowly evaporated on a hot-plate until almost dry. The crucible is then placed in an electric oven, at room temperature, and the furnace temperature raised to 600°. Complete ashing requires from 12 to 24 hours.

The ash is boiled in the crucible on a hot-plate with 15 to 30 cc. of 2

N HCl until completely dissolved, the amount of acid used depending on the quantity of ash. The dissolved ash is evaporated to a volume of about 5 cc. and is then transferred to a 125 cc. Squibb separatory funnel by repeated washing with small portions of hot zinc-free water. When analyzing tissue samples containing more zinc than blood samples, it is desirable to bring the acid-ash solution up to 25 or 50 cc. in a volumetric flask and extract a fraction thereof.

2 cc. of the tartrate solution, together with 2 drops of methyl red, are added to the acid-ash solution in the separatory funnel. Stop-cocks are greased with silicon. The contents of the funnel are then titrated with NH_4OH and H_2SO_4 to pH 5.5, at which methyl red has a peach color. 50 cc. of buffer are added and the contents are allowed to stand until the color has completely faded. Dithizone in CCl_4 (about 10 cc. of a 1 or 10 mg. per cent solution, depending on the amount of zinc present) is added, and the funnel is shaken vigorously for about 2 minutes. The dithizone in CCl_4 solution is allowed to collect in the bottom of the funnel, the last drop is shaken down, and the CCl_4 phase is drawn off into a 50 cc. volumetric flask. This procedure is repeated until the dithizone in the funnel remains a clear green. The sample in the volumetric flask is brought to volume with CCl_4. Depending upon the quantity of excess dithizone present, the final color may be purple or have a greenish tinge.

Colorimetry—Dithizone in CCl_4 has an absorption maximum at 620 mμ; zinc dithizonate has an absorption maximum at 520 mμ, but is transparent at 620 mμ. With the filters employed, the ratio of the relative optical densities of dithizone at 620 and 520 mμ has a numerical value of 4.65 (see Fig. 1).

Readings are obtained on extracted samples at both 520 and 620 mμ with the Evelyn macro photocolorimeter (10). Since the galvanometer used has an optimal accuracy within the range of from 40 to 80 per cent transmission, the final dilution is made with CCl_4 to insure readings falling within this region.

The zinc content of samples is calculated from the equation

$$Z = \frac{L^{520} - \frac{L^{620}}{R} \times D \times K}{\frac{100}{V}} \tag{1}$$

in which Z = total zinc in micrograms in the entire sample; L^{520} = density (2 − log of the galvanometer reading) at 520 mμ; L^{620} = density at 620 mμ; R = the ratio of density of dithizone in CCl_4 at 620 mμ and 520 mμ (numerical value determined as 4.65, see Table I); D = the dilution factor with relation to the original volume (V); K = the calibration constant (numerical value 40; see Table II); V = the volume in which all of the extracted zinc dithizonate is originally diluted.

The ratio of absorption of dithizone at 620 and 520 mμ (R in equation (1)) was determined as follows. A solution of dithizone in CCl_4 was prepared of such a concentration as to deflect the galvanometer to about 10 per cent of the full scale at 620 mμ. A series of dilutions was then made from this so that in the lowest concentration the galvanometer registered about 80 per cent transmission. The series was then read at both wavelengths. The data are given in Table I. In every instance the observed value at 620 mμ was within 2 per cent of the value to be expected from the original concentration and dilution factor. Values for R averaged 4.65 ± 1 per cent.

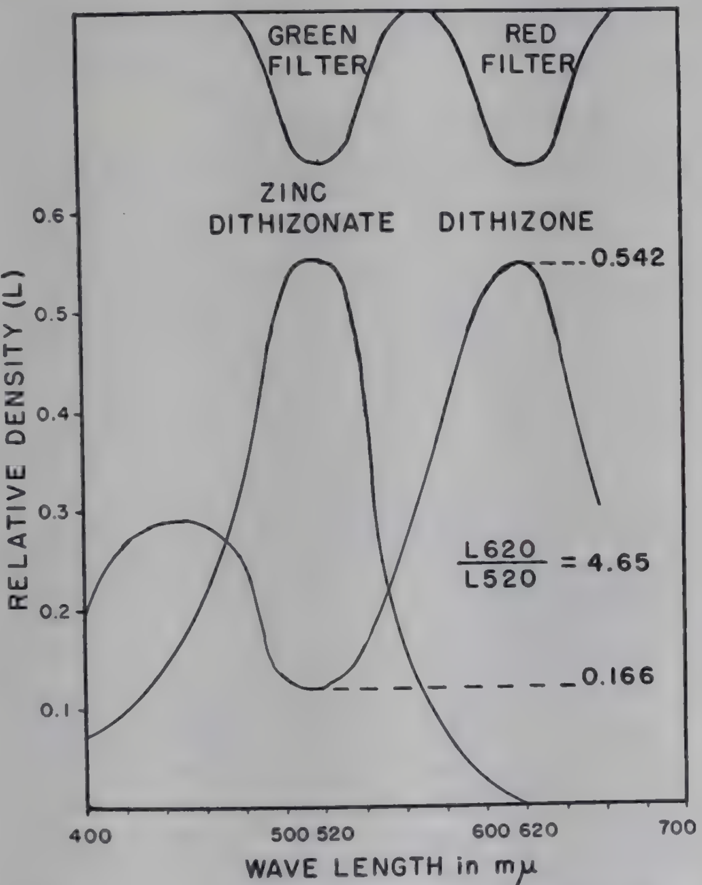


FIG. 1. Absorption curves of dithizone and zinc dithizonate in carbon tetrachloride.

Calibration—A stock solution of zinc chloride was prepared by dissolving 10 mg. of metallic zinc in concentrated HCl and making up to a final volume of 1000 cc. From this solution a series of standards containing from 4 to 50 γ was prepared. These standards were extracted as described above, except that they were not dry ashed. The total zinc dithizonate was made up initially to 50 cc. Further dilutions with CCl_4 were then made to obtain galvanometer readings within the optimal range of the instrument. The data obtained are given in Table II.

TABLE I

Ratio of Absorption of Dithizone at 620 mμ and 520 mμ

Dilution factor	Galvanometer reading	L^{620}			L^{520}	$R = \frac{L^{620}}{L^{520}}$
		Determined	Expected	Deviation		
per cent	*per cent*			*per cent*		
0	10.25	0.989				4.63
0.50	31.75	0.498	0.498	0	0.099	4.67
0.33	47.00	0.328	0.330	−0.6	0.072	4.60
0.33	46.00	0.337	0.330	+2.1	0.072	4.70
0.20	63.75	0.196	0.198	−1.0	0.043	4.63
0.16	69.50	0.158	0.158	0	0.034	4.63
0.10	79.25	0.101	0.099	+2.0	0.021	4.70
Average........						4.65 ± 0.04

TABLE II

Calibration Constant for Zinc Dithizonate-Dithizone Solutions in CCl_4

Total Zn	Dilution factor	L^{520}	L^{620}	$\frac{L^{620}}{4.65}$	Corrected L^{520}	K
γ						
4	0	0.254	0.305	0.066	0.198	40.2
10	2	0.321	0.385	0.083	0.238	42.1
20	3	0.382	0.274	0.058	0.324	41.1
30	4	0.453	0.280	0.062	0.393	38.2
40	5	0.462	0.242	0.052	0.410	39.0
50	5	0.553	0.211	0.045	0.508	39.5
Average........						40.0 ± 2.0

The value for K was calculated from equation (2), derived from equation (1).

$$K = \frac{2Z}{\text{Corrected } L^{520} \times D} \tag{2}$$

The numerical value of K averaged 40.0 ± 2.0, or ±5 per cent.

Inasmuch as this modification of the dithizone method was designed for the analysis of the zinc content of blood samples, it was necessary to determine to what extent the accuracy of the measurement was affected by the dry ashing process.

A stock solution of the $ZnCl_2$ was made up to contain approximately 1 γ of Zn per cc. A series of samples, in duplicate, was prepared containing total quantities of 2, 5, 10, 15, 20, and 30 γ of zinc. These were extracted without dry ashing. The results are given in Table III. The average value of Zn in micrograms per cc. was 0.95, with a standard deviation of 0.05. This was taken as the correct concentration of the stock solution.

An identical series was then dry ashed and extracted, the values obtained also being given in Table III. An average value of 0.97 γ of Zn per cc., with a standard deviation of 0.07, was obtained, indicating that there was no loss of the metal in the ashing. It will be noted that in both series the values for the 2 γ standards were low by about 10 per cent, averaging 0.86 γ per cc. This apparent loss probably is due to slight errors in colorimetry, since similar low values were not obtained in the series described below.

TABLE III

Standardization of Zinc in Samples of Blood Components

Amount analyzed	Stock solution		Stock solution, dry ashed		Dry ashed with white cells, 0.572 γ			Dry ashed with red cells, 3.69 γ			Dry ashed with plasma, 4.29 γ		
cc.	*total* γ	γ *per cc.*	*total* γ	γ *per cc.*	*total* γ	*net* γ	γ *per cc.*	*total* γ	*net* γ	γ *per cc.*	*total* γ	*net* γ	γ *per cc.*
2	1.63	0.81	1.79	0.89	2.75	2.17	1.08	6.39	2.70	1.35	6.71	2.42	1.21
2	1.76	0.88	1.77	0.88	2.31	1.74	0.87	5.53	1.83	0.91	6.11	1.82	0.91
5	4.86	0.97	5.14	1.00	5.46	4.89	0.97	8.54	4.85	0.97	8.91	4.61	0.92
5	4.81	0.96	5.08	0.99	4.92	4.35	0.87	9.02	5.33	1.06	8.68	4.39	0.88
10	9.9	0.99	9.99	0.82	10.57	10.00	1.00	12.37	8.68	0.86	14.08	9.79	0.98
10	9.88	0.98	8.20	1.06				12.59	8.90	0.88			
15	14.80	0.98	16.14	1.05				17.48	13.79	0.92	20.86	16.57	1.10
15	14.36	0.96	15.52	1.00				18.08	14.39	0.96	19.72	15.44	1.00
20	19.27	0.96	20.07	0.99	19.66	19.09	0.96	23.08	18.89	0.93	25.04	20.75	1.00
20	19.00	0.95	19.83	0.99									
30	29.18	0.97	29.88	0.99				31.36	27.67	0.95	30.08	25.79	1.29
30	29.18	0.99	30.21	1.00									
Mean*...		0.95		0.97			0.96			0.98			0.93
S.D., σ....		±0.05		±0.069			±0.08			±0.112			±0.155

* Over-all mean = 0.96; over-all S.D., σ = ±0.0985.

Three additional series were processed, in which known amounts of zinc (2 to 30 γ) were added to samples of separated canine white cells, red cells, and of plasma. In each series the cells and plasma were obtained by floatation of 10 cc. aliquots of the same blood. Individual floatations were done for each standard, and hence the amount of zinc due to cel and plasma was the same at all concentrations of zinc. The total amoun of zinc in the red and white cells and plasma was determined, in duplicate and the average value subtracted from the total zinc found in the severa standards, to obtain the net amount of zinc recovered. Data are give in Table III. The average net zinc recovered (in micrograms per cc. and standard deviation was 0.96 ± 0.08, 0.98 ± 0.112, and 0.93 ± 0.15 γ, in the white cell, red cell, and plasma series, respectively. Thes values compare well with those found for the series that did not contai cells or plasma.

These experiments constitute a check on the over-all accuracy of th procedure, from the separation of cells and plasma from whole blood t the final colorimetric measurement. It would appear that the limit o error lies within ±5 per cent.

As a check on the absence of contamination of all glassware with extraneous zinc, we have found it desirable to determine a 5 or 10 γ standard wit each day's set of extractions. This extraction is carried out with a randon selection of crucibles, separatory funnels, volumetric flasks, pipettes, etc Results obtained in fifteen consecutive analyses of such standards show mean value of 9.82 γ, with a standard deviation of ±0.65. The narrow limits of error found reflect the degree of prevention of contamination i all stages of the procedure.

Since our investigation of zinc metabolism involves the use of the radioactive isotope of zinc, Zn^{65}, it is desirable that measurements of both total and radioactive zinc be made on the same blood or tissue sample. To effect the conversion of zinc dithizonate to a water-soluble zinc salt, *al* of the extracted zinc dithizonate is returned to a clean separatory funnel, a drop of concentrated H_2SO_4 is added, followed by 10 cc. of water, and the funnel is shaken until all the zinc has gone into the aqueous phase, as evidenced by the return of the carbon tetrachloride phase to the green color of dithizone. We have found this method satisfactory in the analysis of blood and tissue samples in dogs (11), in normal humans (12), and in the leucemias,[1] blood dyscrasias, and various other pathological conditions.

SUMMARY

A modification of the dithizone method of extracting zinc from blood and tissue samples is described. The procedure permits of accurate assay of total zinc content of samples in amounts as small as 1 γ.

We wish to acknowledge the technical assistance of Miss Mary L. Roney.

[1] Gibson, J. G., 2nd, and Vallee, B. L., in preparation.

BIBLIOGRAPHY

1. Todd, W. R., and Elvehjem, C. A., *J. Biol. Chem.*, **96**, 609 (1932).
2. Fischer, H., *Wiss. Veröffentl. Siemens-Kongern*, **4**, 158 (1925).
3. Sandell, E. B., Colorimetric determination of traces of metals, New York (1944).
4. Fischer, H., and Leopoldi, G., *Z. anal. Chem.*, **107**, 241 (1936).
5. Holland, E. B., and Ritchie, W. S., *J. Assn. Agr. Chem.*, **22**, 333 (1939).
6. Cowling, H., and Miller, E. J., *Ind. and Eng. Chem., Anal. Ed.*, **13**, 145 (1941).
7. Hove, E., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **136**, 425 (1940).
8. Gettler, A. O., and Bastian, R., *Am. J. Clin. Path.*, **17**, 244 (1947).
9. Vallee, B. L., Hughes, W. L., Jr., and Gibson, J. G., 2nd, *Blood*, Suppl., **1**, 82 (1947).

0. Evelyn, K. A., and Cipriani, A. J., *J. Biol. Chem.*, **117**, 365 (1937).

1. Vallee, B. L., and Fluharty, R. G., *J. Clin. Invest.*, **26**, 1199 (1947).

2. Vallee, B. L., and Gibson, J. G., 2nd, *J. Biol. Chem.*, **176**, 445 (1948).

THE ZINC CONTENT OF NORMAL HUMAN WHOLE BLOOD, PLASMA, LEUCOCYTES, AND ERYTHROCYTES*

By BERT L. VALLEE and JOHN G. GIBSON, 2nd

(From the Medical Clinic of the Peter Bent Brigham Hospital, the Department of Medicine, Harvard Medical School, Boston, and the Department of Physics, Massachusetts Institute of Technology, Cambridge)

(Received for publication, July 11, 1948)

The literature contains few references to the quantitative determination of zinc in human blood. It is difficult to compare the scant data which are available because of the divergence of the methods used and of the units in which different authors chose to express their results (1–5).

It became apparent in the pursuit of our work on zinc in leucocytes and red blood cells, preliminary reports of which have appeared elsewhere (6–8), that the data available in the literature did not offer an adequate point of reference for our studies. A technique was developed (9) for the microanalysis of zinc in biologic materials. This method is sensitive for quantities as small as 1 γ (microgram) and its limits of precision are defined by a standard deviation of ±10.0 per cent. This technique was employed throughout our work.

Arc spectrography[1] of leucocytes and erythrocytes had demonstrated that, for equal numbers of cells, zinc was present in higher concentration in leucocytes than in erythrocytes. We therefore decided to examine whole blood, plasma, washed erythrocytes, and washed leucocytes separately for their zinc content.

Method

Venous blood samples were drawn into a 20 cc. zinc-free syringe (9) which had previously been wetted with heparin. Red and white blood cell counts were done in standard hemocytometers, in duplicate. Only counts agreeing within 5 per cent were accepted. Hematocrits were carried out in Wintrobe tubes, spinning 30 minutes at 3000 R.P.M. Hemoglobin was determined by the method of Evelyn (10). Differential counts of leucocytes were made from smears stained with Wright's stain, 300 cells being counted.

A separation of red cells, white cells, and plasma was performed by the

---

* This study was supported by a grant-in-aid from the National Institute of Health.

[1] Through the courtesy of Dean George R. Harrison and Mr. Rockwell Kent, 3rd, Massachusetts Institute of Technology.

flotation technique (11). All of the separated leucocytes were removed from the flotation tube and washed in a 0.02 per cent saponin solution in 0.85 per cent NaCl, until all entrained red cells had been removed, as evidenced by the absence of hemoglobin in the supernatant washing. Zinc analysis was carried out on the entire mass of washed white cells.

A portion of the red blood cells was transferred from the flotation tube to a centrifuge tube and washed in 0.85 N saline by centrifugation. The washing was discarded, and an aliquot of the packed cells was transferred to a 4 cc. hematocrit tube and made up to volume with 0.85 per cent NaCl solution. The quantity of cells was such that the final hematocrit was about 30. Two further washings were performed, in the 4 cc. tube, and from the third and final dilution a red blood count (in duplicate), hematocrit reading, and hemoglobin determination were obtained. The duplicate red blood counts were required to check within ±5 per cent. These 4 cc. samples of red blood cell suspensions were the final aliquots used for analysis.

The measurement of the zinc content of whole blood, plasma, leucocyte, and erythrocyte samples was performed as previously described (9). Routinely 2 cc. of whole blood and 3 cc. of plasma were analyzed.

Calculation of Results—Zinc concentrations in whole blood and plasma were expressed in micrograms per cc. analyzed. Since volumetric measurement of white cells (by hematocrit) was impractical, due to the limited mass of white cells obtainable and the large variation in dimensions of the various classes of white cells, unit zinc concentrations for leucocytes were calculated in micrograms per million cells. Unit zinc concentrations for red cells also were calculated in micrograms per million cells for comparison with unit white cell zinc concentrations, and in micrograms per cc. of packed cells. Examples of calculations of results for leucocytes and erythrocytes follow.

The total number of cells in the sample of leucocytes was determined by multiplying the white cell count per c.mm. by the number of c.mm. of whole blood floated. For example, the white blood cell count $= 1 \times 10^4$ cells per c.mm.; whole blood floated = 15 cc.; total cells in 15 cc. of whole blood $= 1 \times 10^4 \times 1 \times 10^3 \times 1.5 \times 10^1 = 150 \times 10^6$ cells; total Zn in sample $= 3.0\ \gamma$; Zn per 1×10^6 cells $= 2 \times 10^{-2}\ \gamma$.

The total number of red cells in the sample was determined by multiplying the red count of the final dilution of cells by the final volume of that suspension. For example, the red blood cell count on final red cell dilution $= 3.5 \times 10^6$ per c.mm.; total cell suspension analyzed = 4.0 cc.; thus $3.5 \times 10^6 \times 1 \times 10^3 \times 4 = 14{,}000 \times 10^6$ cells; total Zn in sample $= 17.5\ \gamma$; Zn per 1×10^6 cells $= 1.25 \times 10^{-3}\ \gamma$.

The zinc in cc. of packed cells was calculated on the basis of micrograms of zinc per million cells, red blood cell count, and hematocrit of whole blood, as follows: the red blood cell count on whole blood (undiluted) $= 5 \times 10^6$ per c.mm.; hematocrit of whole blood = 45.0 per cent; thus $(1.25 \times 10^{-3} \times 5 \times 10^6 \times 1 \times 10^3)/(4.5 \times 10^{-1}) = 13.9\ \gamma$ per ml. of packed cells.

As a check on the accuracy of the individual measurements of whole blood, and the three blood components analyzed, an internal check was calculated for each blood sample. The amount of zinc in 1 cc. of whole blood due to the zinc content of white cells, red cells, and of plasma was calculated as follows: (total Zn in all white cells analyzed)/(15 cc.) = Zn in 1 cc. of whole blood due to leucocytes; ((Zn)/(million red blood cells)) × ((red blood cells)/(cc. of whole blood)) = Zn in 1 cc. of whole blood due to erythrocytes; Zn in 1 cc. of plasma × (1 − (hematocrit)/100) = Zn in 1 cc. of whole blood due to plasma; = total Zn in 1 cc. of whole blood.

The deviation of the total whole blood zinc so calculated and the directly determined zinc content of whole blood was taken to be a measure of the over-all accuracy of the hematological procedures and of the preliminary processing, including dry ashing, as well as the chemical analyses for zinc.

Material Studied—Thirty-one normal individuals, fifteen males and sixteen females, ranging in age from 18 to 45 years, were studied. Repeated determinations were carried out in six of these subjects, bringing the total of single analyses to thirty-eight. All blood samples were drawn in the morning with the subject fasting but not otherwise under basal conditions. Each sample was analyzed for all of the data described above under "Method." In a few instances one of the components was lost in processing.

In addition, five patients, with no apparent blood dyscrasias, on the medical wards of the Peter Bent Brigham Hospital, were studied. One had a peptic ulcer, one had recovered from bronchopneumonia, one had milkman's disease, one was compensating from congestive heart failure, and one was suspected of having a brain tumor.

Fifteen consecutive blood samples taken at 1 to 4 day intervals from the same individual, Case 53, a 22 year-old normal male, were also analyzed.

Results

Unit zinc content, as described above, for whole blood and its three components for the group of normals and the patients is given in Table I, and for the series in one normal person, in Table II. Also shown in Tables I to IV are the means and standard deviations (S.D.) for all unit zinc concentrations.

The values for the amount of zinc contained in 1 cc. of whole blood due to plasma, leucocytes, and erythrocytes, calculated as described above, are shown for the normals and patients in Table III, and for Case 53 in

TABLE I

Unit Values of Zinc Content of Whole Blood, Plasma, Leucocytes, and Erythrocytes

Experiment No.	Date	Unit content by dithizone extraction				
		Whole blood	Plasma	Leucocytes	Erythrocytes	
Normal males						
	1947	*γ per cc.*	*γ per cc.*	*$\gamma \times 10^{-2}$ per 1×10^{6} cells*	*$\gamma \times 10^{-8}$ per 1×10^{6} cells*	*Zn γ per cc. packed red cells**
23-1	May 9	7.7	2.6	1.2	1.21	13.2
23-2	" 12	9.2	2.2	4.2	1.35	14.7
29-1	" 16	7.6	1.2	1.6	1.25	16.4
34-1	" 26	10.5	4.3	2.9	1.41	15.7
36-1	" 28	7.5	3.2	4.6	1.20	12.6
73-1	Sept. 11	13.7	11.4	2.8	1.20	11.9
80-1	" 29	5.9	2.0	2.2	0.97	9.1
80-2	Oct. 8	8.1	3.7	6.5	1.20	12.3
84-1	" 2	6.3	1.9	2.0	1.30	12.7
85-1	" 2	7.0	3.0	1.8	1.15	12.2
88-1	" 6	9.5	4.1	1.9	1.71	17.0
88-2	" 20	7.9	1.7	2.1	1.19	15.2
89-1	" 6	8.3	3.2	4.7	1.44	16.6
90-1	" 7	8.6	4.1	2.3	1.20	14.1
99-1	" 16	9.1	5.5	1.4	1.28	13.6
108-1	" 24	9.2	3.8	5.5	1.59	17.1
109-1	" 24	14.8	9.1	2.3	1.83	18.3
122-1	Nov. 19	9.3	3.7		1.29	18.4
Mean.......		8.9	3.9	2.9	1.31	14.2
S.D..........		±2.0	±2.5	±1.5	±0.25	±2.3
Normal females						
24-1	May 12	9.1	3.0	4.8	1.26	13.8
25-2	June 26	8.5	3.4	3.8	1.32	13.6
25-3	Sept. 26	7.9	2.2	2.6	1.36	15.0
26-1	May 14	8.5	1.6	3.5	1.06	11.3
28-1	" 14	9.1	2.0	2.9	1.15	13.5
28-2	Oct. 3	6.7	1.3	6.2	1.30	13.6
31-1	May 19	8.7	2.0	4.2	1.27	13.8
76-1	Sept. 18	5.2	1.5		1.09	16.1
77-1	" 18	7.6	1.2	1.7	1.21	12.5
82-1	Oct. 1		4.4	3.6	1.60	16.7
86-1	" 3		4.8	4.4	1.63	17.1
86-2	" 9	8.1	3.2	2.1	1.38	13.9
86-3	Nov. 5	13.2	10.1	3.1	1.54	16.5
87-1	Oct. 3		4.5	2.9	1.42	12.8
93-1	" 9	11.5	4.6	3.1	1.54	17.0

TABLE I—*Concluded*

Experiment No.	Date	Unit content by dithizone extraction				
		Whole blood	Plasma	Leucocytes	Erythrocytes	
Normal females—*continued*						
	1947	*γ per cc.*	*γ per cc.*	*$\gamma \times 10^{-2}$ per 1×10^6 cells*	*$\gamma \times 10^{-3}$ per 1×10^6 cells*	*Zn γ per cc. packed red cells**
100-1	Oct. 17	7.2	2.3	3.5	1.24	11.9
101-1	" 17	8.6	3.0	4.2	1.32	14.2
115-1	Nov. 4	9.4	4.2	1.9	1.17	12.9
116-1	" 5	10.4	4.1	4.6	1.85	18.4
123-1	" 19	8.4	1.2		1.52	15.5
Mean.......		8.6	3.1	3.5	1.37	14.5
S.D..........		±1.8	±2.0	±1.2	±0.195	±1.9
Total normals						
Mean.......		8.8	3.0	3.2	1.34	14.4
S.D..........		±2.0	±1.6	±1.3	±0.20	±2.7
Patients with no blood dyscrasias						
30-1 M.	May 16	8.6	2.7	6.5	1.16	13.8
48-1 "	June 17	8.1	4.4	4.3	1.28	13.3
40-1 F.	" 4	8.5	4.2	2.0	1.43	14.9
50-1 "	July 21	12.3	8.7	2.4	1.33	14.6
61-1 "	Aug. 14	9.6	8.2	1.6	1.41	16.0
Mean.......		9.0	5.6	3.2	1.32	14.5

* Corrected for mean corpuscular volume.

Table IV. The total of the three values constitutes the "reconstructed" total blood zinc content.

DISCUSSION

In the evaluation of the data presented above, the limitations of the various hematological techniques employed must be taken into consideration. The errors inherent in the chemical analyses for zinc have been previously discussed (9).

Since Ostwald pipettes were used for the whole blood and plasma samples, little additional error was introduced in the preparation of these samples for chemical analysis.

The limited quantity of leucocytes obtained from a routine sample of 15 cc. of blood precluded making a volumetric measurement on these cells. It was not possible to make a final count on the leucocytes separated from heparinized blood because of clumping of cells, which persisted even after repeated washing with the saponin-saline solution. The "million cell unit" obviously reflects the errors inherent in leucocyte counts carried out in standard hemocytometers, although this error was reduced by duplicate counting. However, this unit allows conclusions as to the zinc content of individual white cells, and is therefore of value in the study of clinical conditions affecting leucopoiesis, and for comparison with corresponding values for erythrocytes. The development of better techniques for handling leucocytes is now in progress.

TABLE II

Unit Values of Zinc Content of Whole Blood, Plasma, Leucocytes, and Erythrocytes in Normal Individual (Case 53)

Fifteen consecutive samples.

Experiment No.	Date	Unit content by dithizone extraction				
		Whole blood	Plasma	Leucocytes	Erythrocytes	
	1947	*γ per cc.*	*γ per cc.*	*γ × 10⁻³ per 1 × 10⁶ cells*	*γ × 10⁻³ per 1 × 10⁶ cells*	*Zn γ cc. packed red cells**
1	July 31	8.4	3.3	1.2	1.48	15.7
2	Aug. 4	8.3	5.2	2.2	1.37	14.5
3	" 4	9.3	6.0	1.7	1.63	17.9
4	" 5	8.7	4.4	2.0	1.43	15.0
5	" 6	7.7	2.7	4.2	1.20	11.7
6	" 7	11.1	4.2	2.0	1.77	18.7
7	" 8	9.1	3.6	5.1	1.51	15.9
9	" 12	9.9	4.8	2.0	1.40	15.1
10	" 13	8.5	2.8	2.0	1.69	16.4
11	" 14	8.4	2.8		1.66	16.5
12	" 15	10.2	5.6	1.9	1.66	17.2
13	" 18	9.4	3.6	1.5	1.14	12.5
14	" 19	9.9	2.6	3.5	1.44	16.5
17	" 22	7.6	1.5	1.9	1.19	12.5
19	" 27	9.5	5.0	3.7	1.59	17.1
Mean..........		9.1	3.9	2.5	1.47	15.5
S.D............		±0.9	±1.3	±1.1	±0.18	±0.6

* Corrected for mean corpuscular volume.

The use of the million cell unit for erythrocytes also involves the same sources of error in about the same degree. However, the calculation of unit zinc content of red cells on the basis of hematocrit and red count permits comparison of unit zinc concentrations in anemias and other disturbances of erythropoiesis with the normal.

Inspection of the data in Tables I and II shows that the values for whole blood, plasma, leucocytes, and erythrocytes in males, females, and in the totals vary over a fairly wide range. The measurements performed on one individual (Case 53) are of the same order of magnitude as found in the series of normals, though both their range and standard deviation are much narrower. They occupy the central region of a hypothetical distribution curve plotted for the data obtained from the series.

The variations and standard deviations of the white blood cell measurements are greater than those observed in the other categories. This could be predicted from the technical limitations imposed by the processing of leucocytes, as described above. Furthermore, the total zinc content of a leucocyte sample is of necessity small, because of the small number of white cells in circulation.

The larger aliquots of whole blood, plasma, and red cells obtainable for routine analysis contain about 5 times as much zinc as is contained in all the white cells from 15 cc. of whole blood. Since the percentage error of zinc extraction tends to be constant, the absolute error will of necessity be greater for white cells than in the case of whole blood, plasma, or red cells.

The distribution curves shown in Fig. 1 graphically express these facts, and are plotted from data obtained in normals given in Table III. The incidence is plotted for males, females, and total cases. Fig. 1, *A* is the curve for the directly measured whole blood zinc content. The curve is symmetrical and the mode closely approximates the arithmetic mean.

The distribution curves for plasma, Fig. 1, *B*, for leucocytes, Fig. 1, *C*, and for erythrocytes, Fig. 1, *D*, are plotted, not from the directly measured unit values, but from the "reconstructed" value of zinc in 1 cc. of whole blood due to the particular component given in Table III. It was felt that the latter values more closely represented a fractional analysis of blood than did the direct determinations.

The curve for plasma, though nearly symmetrical, appears to be slightly skewed to the left. It will be noted that three of the cases fall well to the positive side of the mean. There was a reasonable probability that these values may have been high due to accidental contamination with zinc but, since the evidence is not conclusive, they are included in the series. The inclusion of these three cases raises the mean. If these are omitted, the mean falls to 1.53 γ per ml., whereas the mode is approximated at 1.25. Thus biological significance cannot be attributed to the skewness of the curve.

The curve for leucocytes is grouped around a mean value of 0.21 γ per ml., with a mode of 0.170. Here again the mean is slightly higher due to the inclusion of two cases with values much greater than the mean value plus twice the standard deviation. It should be stated that the absolute quantities of zinc measured in the samples are extremely small, so that losses in processing, which would not show up in samples of whole blood or of the other components, could be sufficient to account for the predominantly low values found in the majority of cases.

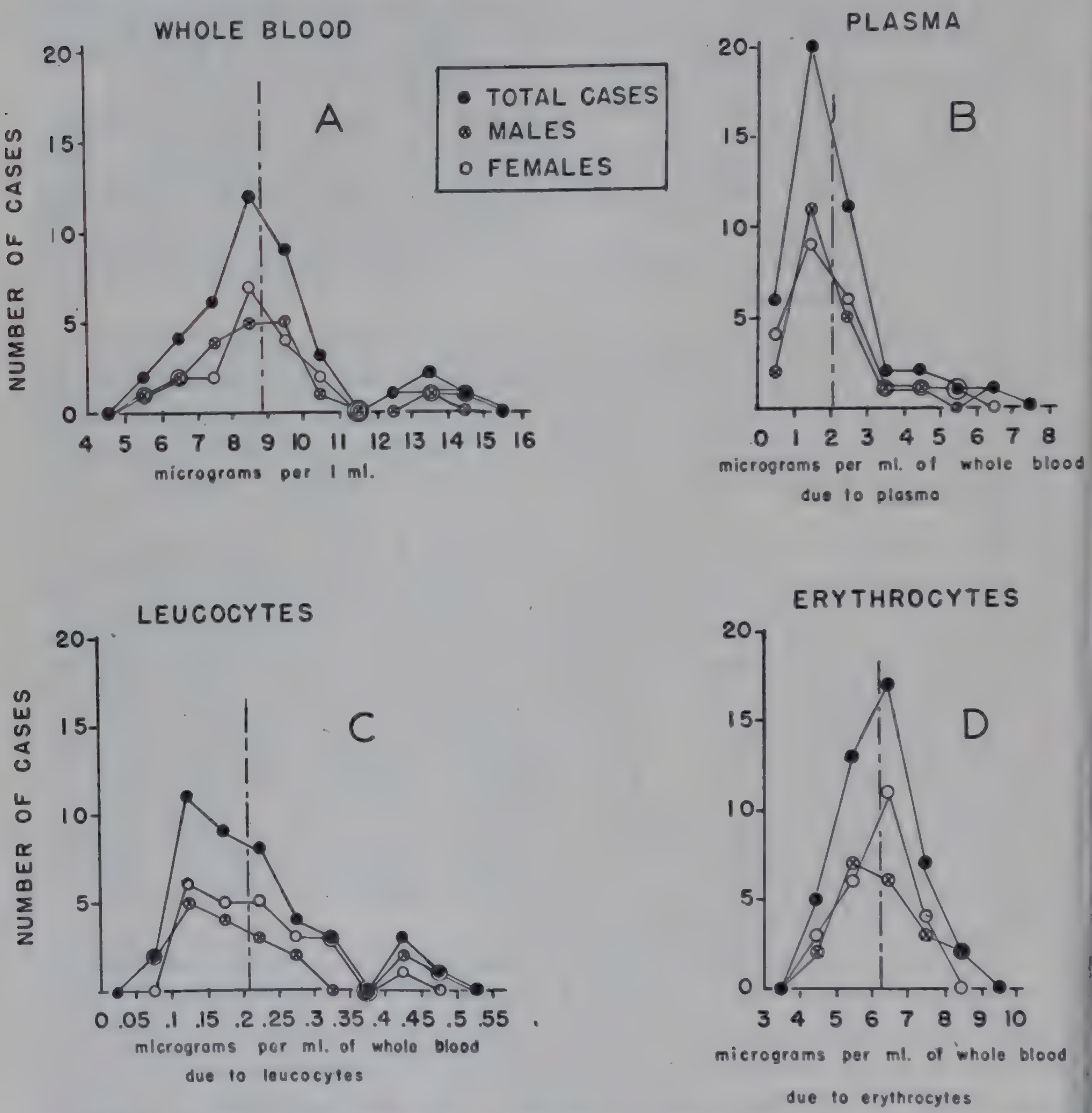


FIG. 1. Distribution curves of zinc content of whole blood, plasma, leucocytes, and erythrocytes in normals.

The distribution curve for red cells, Fig. 1, *D*, is symmetrical and shows little skewness.

It is apparent that no significant difference exists between sexes in this series.

TABLE III

Zinc Content of 1 Cc. of Normal Whole Blood Calculated from Unit Values of Plasma, Leucocytes, and Erythrocytes

All values in micrograms.

Experiment No.	Plasma	Leucocytes	Erythrocytes	Total Zn in 1 cc. of whole blood		Per cent deviations
	(1)	(2)	(3)	(4)	(5)	(6)
			Normal males			
23-1	1.4	0.08	6.2	7.7	7.7	0
23-2	1.2	0.33	6.8	8.4	9.2	−8.7
29-1	0.7	0.11	5.8	6.6	7.6	−13.2
34-1	2.4	0.20	6.9	9.5	10.5	−9.5
36-1	1.8	0.29	5.6	7.7	7.5	+2.7
73-1	6.2	0.22	5.5	12.0	13.7	−12.4
80-1	1.2	0.08	4.0	5.3	5.9	−10.3
80-2	2.3	0.20	4.6	7.1	8.1	−12.3
84-1	1.0	0.15	5.7	6.9	6.3	+9.5
85-1	1.5	0.14	5.9	7.5	7.0	+7.2
88-1	2.0	0.17	8.9	11.1	9.5	+16.9
88-2	0.8	0.19	7.5	8.5	7.9	+7.6
89-1	1.6	0.25	7.6	9.4	8.3	+13.3
90-1	2.3	0.25	6.4	9.0	8.6	+4.7
99-1	3.1	0.12	5.9	9.1	9.1	0
108-1	2.1	0.47	7.7	10.3	9.2	+12.0
109-1	4.9	0.15	8.4	13.5	14.8	−8.9
122-1	1.4		6.1		9.3	
Mean.....	2.10	0.20	6.4	8.8	8.9	−0.1
S.D........	±1.40	±0.095	±1.25	±2.03	±2.0	±10.0
			Normal females			
24-1	1.8	0.32	5.6	7.7	9.1	−15.4
25-2	1.8	0.32	6.0	8.1	8.5	−4.7
25-3	1.2	0.18	6.6	8.0	7.9	+1.3
26-1	0.9	0.24	4.7	5.8	8.5	−31.8
28-1	1.1	0.19	6.2	7.5	9.1	−17.6
28-2	0.6	0.47	6.0	7.1	6.7	+6.0
31-1	1.1	0.23	6.0	7.3	8.7	−16.1
76-1	1.2	0.13	6.4	7.7	5.2	+48.2
77-1	0.7	0.13	5.7	6.5	7.6	−14.5
82-1	2.5	0.22	7.4	10.1		
86-1	3.1	0.22	6.1	9.4		
86-2	1.9	0.14	5.6	7.6	8.1	−6.2
86-3	6.0	0.17	6.8	13.0	13.2	−1.5
87-1	2.5	0.14	5.3	7.9		
93-1	2.5	0.26	7.8	10.6	11.5	−7.8

TABLE III—*Concluded*

Experiment No.	Plasma	Leucocytes	Erythrocytes	Total Zn in 1 cc. of whole blood		Per cent deviations
	(1)	(2)	(3)	(4)	(5)	(6)
Normal females—*continued*						
100-1	1.4	0.17	4.9	6.5	7.2	−9.7
101-1	1.7	0.30	5.6	7.6	8.6	−11.7
115-1	2.4	0.13	5.7	8.2	9.4	−12.8
116-1	2.5	0.30	7.9	10.7	10.4	+2.9
123-1	0.6		7.0		8.4	
Mean.....	1.80	0.22	6.2	8.2	8.6	−5.7
S.D........	±1.10	±0.09	±0.83	±1.65	±1.8	±16.4
Total normals						
Mean.....	2.05	0.21	6.3	8.5	8.8	−2.9
S.D........	±1.30	±0.09	±1.05	±1.84	±2.0	±14.3
Patients with no blood dyscrasias						
30-1 M.	1.6	0.43	5.9	7.9	8.6	−8.1
48-1 "	2.2	0.44	6.5	9.1	8.1	+12.4
40-1 F.	2.5	0.17	6.1	8.8	8.5	+3.5
50-1 "	4.6	0.21	7.0	11.8	12.3	−4.1
61-1 "	1.9	0.13	6.5	8.5	9.6	−11.5
Mean.....	2.56	0.28	6.4	9.3	9.4	−1.7

Column 1, (Zn per cc. of plasma × 100) − (hematocrit of whole blood); Column 2, (total zinc in sample) ÷ (cc. of whole blood from which leucocytes were obtained); Column 3, Zn per million red cells × red cell count of whole blood × 1 × 10^3; Column 4, sum of Columns 1, 2, and 3; Column 5, zinc content of 1 cc. of whole blood directly measured; Column 6, percentage difference between Columns 5 and 4.

The shapes of the curves for whole blood and the three components thereof indicate that the data presented are a measure of a biological norm, variable within physiological limits.

This fact is affirmed by the determinations carried out on the individual (Case 53) shown in Table IV. The mean values of the fractions and total blood zinc were close to those of the normal series but varied within much narrower limits, as indicated by the standard deviation.

Data on five patients who were suffering from illnesses other than blood dyscrasias are included, with their mean values. No standard deviation was derived because of the small number of patients involved. It will be noted that the unit zinc values lie within the range of values in the normal series.

The percentage deviation in the reconstructed total ranged from +48.2 to −31.8, averaging −2.9, S.D. ±14.3 for the normal series. The range for the five patients was +12.4 to −11.5, averaging −1.7. The range in Case 53 was from +20.0 to −17.0, averaging +2.2, S.D. ±11.0.

For statistical evaluation of the data, we have assumed that the direct determination on whole blood represents 100 per cent recovery of zinc.

TABLE IV

Zinc Content of 1 Cc. of Whole Blood Calculated from Unit Values of Plasma, Leucocytes, and Erythrocytes in Normal Individual

Case 53, normal 22 year-old male; fifteen consecutive samples; all values in micrograms.

Experiment No.	Plasma	Leucocytes	Erythrocytes	Total Zn in 1 cc. of whole blood		Per cent deviations
	(1)	(2)	(3)	(4)	(5)	(6)
1	1.9	0.10	6.7	8.7	8.4	+3.6
2	2.7	0.19	7.0	9.9	8.3	+17.0
3	3.3	0.15	7.8	11.2	9.3	+20.0
4	2.4	0.19	6.7	9.3	8.7	+6.9
5	1.6	0.29	5.2	7.1	7.7	−7.8
6	2.4	0.19	8.1	10.7	11.1	−3.5
7	2.0	0.35	7.0	9.4	9.1	+4.4
9	2.7	0.25	6.6	9.5	9.9	−4.1
10	1.6	0.16	7.3	9.1	8.5	+7.1
11	1.5		7.4		8.4	
12	3.1	0.15	7.6	10.8	10.2	+5.9
13	1.9	0.14	5.8	7.8	9.4	−17.0
14	1.4	0.23	7.2	8.8	9.9	−11.1
17	0.9	0.17	5.5	6.6	7.6	−13.2
19	2.7	0.25	7.8	10.8	9.5	+13.7
Mean.....	2.14	0.20	6.9	9.2	9.1	+2.2
S.D.......	±0.56	±0.06	±0.88	±1.34	±0.92	±11.0

Explanation of columns the same as in Table III.

The mean of the reconstructed total (Column 4, Table III) is slightly lower than the mean of the direct measurements.

The mean of the reconstructed whole blood zinc in Case 53 is slightly higher than the mean of the direct measurements.

The percentage deviation of Column 4 from Column 5, listed in Column 6 (Tables III and IV), brings these facts out more clearly. The mean percentage deviation of the series is −2.9 ± 14.3, and for Case 53 (Table IV), the mean is +2.2 ± 11.0. Therefore the reconstructed whole blood zinc level may be stated to fall within about ±15 per cent of the zinc concentration found for whole blood by direct measurement.

It should be stated that the large range of percentage deviations actually represents minute differences in the absolute quantity of zinc present in the materials measured. Thus a loss or gain of 0.5 γ from a total of 5 γ is ±10 per cent, whereas the same quantity would constitute only ±5 per cent of the total of 10 γ. This statistical treatment of the data was carried through to show the inherent limitations of the technique. In our opinion, however, the method appears to be useful in clinical investigation.

Normally, red blood cell zinc constitutes 75 per cent, white blood cells 3 per cent, and plasma 22 per cent of total whole blood zinc. The greater portion of whole blood zinc is red cell zinc. However, the individual leucocyte contains about 25 times as much zinc as the individual erythrocyte.

The data available at present do not disclose the possible differences in the zinc content of the various classes of leucocytes. Such studies must await the development of techniques for obtaining pure specimens of the lymphoid and myelogenous series of white cells.

The findings presented suggest that zinc is a physiological constituent of blood, and its individual variations in concentration follow the mathematical pattern of commonly observed biological distribution phenomena.

The normal values here reported will serve as a base for comparison of the results of studies of blood zinc levels in the blood dyscrasias, now in progress.

Inasmuch as these studies employ the combined use of the chemical analyses and a radioactive tracer, Zn^{65}, the data herein reported will serve as a basis for the study of the utilization of zinc in hematopoiesis, and the transport and distribution of zinc in the normal state and in disease.

SUMMARY

1. The zinc content of normal human whole blood, plasma, leucocytes, and erythrocytes in males and females has been determined.

2. Red blood cell zinc constitutes 75 per cent, plasma zinc 22 per cent, and white cell zinc 3 per cent of whole blood zinc in normal human blood. The individual leucocytes contain about 25 times the amount of zinc found in erythrocytes.

3. Zinc concentration in blood and its components follows the distribution pattern of physiological norms.

We wish to acknowledge the technical assistance of Miss Mary L. Roney, Miss M. Elizabeth Hickey, and Miss Barbara M. Clapp.

BIBLIOGRAPHY

1. Delezenne, C., *Ann. Inst. Pasteur*, **33**, 68 (1919).
2. Lutz, R. E., *J. Ind. Hyg.*, **8**, 177 (1926).

3. Burstein, A. I., *Biochem. Z.*, **216**, 449 (1929).
4. Vogelenzang, E. H., *Chem. Abstr.*, **33**, 6435 (1939); *Pharm. Weekbl.*, **76**, 89 (1939).
5. Eggleton, W. G. E., *Chinese J. Physiol.*, **15**, 33 (1940).
6. Vallee, B. L., and Fluharty, R. G., *J. Clin. Invest.*, **26**, 1199 (1947).
7. Vallee, B. L., Fluharty, R. G., and Gibson, J. G., 2nd, 4th International Cancer Research Congress, St. Louis, Sept. 2–7 (1947).
8. Gibson, J. G., 2nd, Vallee, B. L., Fluharty, R. G., and Nelson, J. E., 4th International Cancer Research Congress, St. Louis, Sept. 2–7 (1947).
9. Vallee, B. L., and Gibson, J. G., 2nd, *J. Biol. Chem.*, **176**, 435 (1948).
10. Evelyn, K. A., *J. Biol. Chem.*, **115**, 63 (1936).
11. Vallee, B. L., Hughes, W. L., Jr., and Gibson, J. G., 2nd, *Blood*, Suppl., **1**, 82 (1947).

LETTERS TO THE EDITORS

THE ACETYL PRECURSOR IN PYRUVATE SYNTHESIS IN ESCHERICHIA COLI*

Sirs:

The double function of acyl phosphate as a phosphate and acyl donor is indicated by a great variety of experiments.[1] Only the phosphate donor function, however, has been observed in a chemically well defined compound as synthetically prepared monoacetylphosphate.[2,3] Enzymatic acyl transfer so far had been found only with acetate plus ATP,[4,5] but not with the corresponding acyl phosphate.[5,6] Nevertheless, a reverse transfer of phosphate from ATP to acetate and butyrate had been observed with bacterial extracts.[2] It now appears that this reverse reaction is at least partially not a true reversal.

A more extensive study of the reaction between ATP and acetate became promising with the availability of the hydroxamic acid method for acyl phosphate determination.[7] With this method, a rather active phosphorylation of acetate with ATP was found in extracts of dried *E. coli*, particularly with high concentration of acetate. Some differences between the product of this reaction and synthetically prepared acetyl phosphate have been described previously.[3] The product has now been partially purified, and fractions were obtained containing equivalent amounts of reactive acetyl and labile phosphate but little or no stable phosphate. By short exposure to pH 1.5 or less even at room temperature, it becomes indistinguishable from synthetic acetyl phosphate. It appears possible that the compound is an acetyl phosphate derivative. It is, however, not diacetylphosphate.

The ATP-acetate reaction product reacts as an acetyl donor in the synthesis of pyruvate in dialyzed suspensions of dried *E. coli*. Grossly, this reaction is the reversal of the phosphoroclastic split of pyruvate to acetyl phosphate and formate. Synthetic acetyl phosphate reacts only in the

* Aided by a grant from the Commonwealth Fund.

[1] Lipmann, F., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **6**, 231 (1946).

[2] Lipmann, F., *J. Biol. Chem.*, **155**, 55 (1944).

[3] Kaplan, N. O., and Lipmann, F., *Federation Proc.*, **7**, 163 (1948).

[4] Utter, M. F., Lipmann, F., and Werkman, C. H., *J. Biol. Chem.*, **158**, 521 (1945).

[5] Lipmann, F., *J. Biol. Chem.*, **160**, 173 (1945).

[6] Strecker, H., Krampitz, L. O., and Wood, H. G., *Federation Proc.*, **7**, 194 (1948).

[7] Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159**, 21 (1945).

presence of ADP, presumably as a phosphate donor (see the table). ATP plus acetate with formate present, however, causes an easily measurable synthesis of pyruvate. These results confirm earlier observations of Utter, Lipmann, and Werkman[4] of synthesis of carbonyl-marked pyruvate from carboxyl-marked acetate plus ATP. With the isolated reaction product of ATP and acetate, an almost quantitative synthesis to pyruvate was found with excess formate. The preparation used in this experiment was free of ATP, and contained only traces of other organic phosphates. The further purification and identification of the reaction product are in progress.

1 cc. of dialyzed, 10 per cent dried *E. coli* suspension, 0.1 cc. of 0.1 M $MgCl_2$, and 20 micromoles of cocarboxylase added to each tube. Total volume 4 cc., incubated at 37° for 30 minutes. Pyruvate was determined by the Friedemann-Haugen method (*J. Biol. Chem.*, **147**, 415 (1943)).

Additions	Micromoles added	Acetate, micromoles added	Formate, micromoles added	Pyruvate formed, micromoles per cc. of extract
Synthetic diacetyl phosphate	20	1000	500	0
" monoacetyl phosphate	25	1000	500	0
ADP	20	1000	500	1.4
"	20			
Synthetic monoacetyl phosphate	25	1000	500	2.2
ATP	25	1000	500	3.9
"	25	1000		1.0
ATP-acetate reaction product	3		500	2.8
" " "	6		500	4.8

Biochemical Research Laboratory, Massachusetts General Hospital, and the Department of Biological Chemistry, Harvard Medical School
Boston

Nathan O. Kaplan
Fritz Lipmann

Received for publication, July 26, 1948

THE CONVERSION OF 16-KETOESTRONE TO ESTRIOL IN VIVO*

Sirs:

The hypothesis has been advanced that 16-ketoestrone is an intermediate in the metabolism of estrone to estriol.[1] To gain further knowledge concerning the metabolic fate of 16-ketoestrone, 600 mg. of this steroid dissolved in propylene glycol were administered intramuscularly in divided doses of 10 mg. per cc. to a man. The pooled urines (18.0 liters) were collected under toluene and subsequently hydrolyzed by boiling with 10 volumes per cent of hydrochloric acid for 20 minutes. Aliquots (1.5 liters) of the hydrolyzed urine were processed, chromatographed, and assayed by previously described methods.[2] From the combined "estriol" fractions a semicrystalline material was obtained which gave a titer of 10.72 and 10.95 mg. of estriol with the Kober and Bachman reagents respectively. In each instance the spectrum was typical of the color product produced by pure estriol. Furthermore, 16-ketoestrone gave no characteristic color with either of these reagents and 16-keto-α-estradiol gave a characteristic color with only one of them (the Kober reagent). The semicrystalline material was further purified by solvent partition according to Friedgood and Garst[3] by use of benzene and 0.075 M Na_2HPO_4, and then separated into *cis*-oid and *trans*-oid steroidal 16,17-glycols.[4] From the *trans*-oid portion after recrystallization from acetone-cyclohexane and aqueous methanol (charcoal), 2.4 mg. of crystals, melting at 267–269°, uncorrected, were obtained. These crystals showed no melting point depression on admixture with authentic theelol[5] (m.p. 268.5–269.5°, uncorrected). A very careful comparison[6] of the estrogenic titers of authentic theelol and the isolated crystals in the Curtis-Doisy assay[7] (with a total of 120 immature female rats)

---

* This work was supported in part by a grant to the Rees-Stealy Medical Research Fund from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council, and was supported in part by a grant from the United States Standard Products Company, Woodworth, Wisconsin.

[1] Huffman, M. N., *J. Am. Chem. Soc.*, **64**, 2235 (1942). Huffman, M. N., and Grollman, A., *Endocrinology*, **41**, 12 (1947).

[2] Stimmel, B. F., *J. Biol. Chem.*, **162**, 99 (1946).

[3] Friedgood, H. B., and Garst, J. B., Recent progress in hormone research, New York, **2**, 31 (1948).

[4] Huffman, M. N., and Lott, M. H., *J. Am. Chem. Soc.*, **69**, 1835 (1947).

[5] Kindly supplied by Dr. D. W. MacCorquodale of the Abbott Laboratories, North Chicago, Illinois.

[6] We wish to thank Mr. Clyde J. Newton of the Magnolia Field Research Laboratories, Dallas, for weighing on the microbalance a sample of the isolated estriol for assay purposes.

[7] Curtis, J. M., and Doisy, E. A., *J. Biol. Chem.*, **91**, 647 (1931).

showed identical values. There seems, therefore, no doubt that the human organism can convert 16-ketoestrone to estriol.

Rees-Stealy Medical Research Fund
San Diego, California
Departments of Pharmacology, Experimental Medicine, and Biochemistry
Southwestern Medical College
Dallas

BENJAMIN F. STIMMEL
ARTHUR GROLLMAN
MAX N. HUFFMAN[8]

Received for publication, August 18, 1943

---

[8] One of us (M. N. H.) would like to acknowledge the technical assistance of Miss Mary Harriet Lott and Mr. James Ashmore.

ADAPTIVE CONVERSION OF MALATE TO LACTATE AND CARBON DIOXIDE BY LACTOBACILLUS ARABINOSUS*

Sirs:

Washed resting cells of *L. arabinosus* (strain 17-5) evolve little CO_2 from *l*-malic acid anaerobically when harvested from media containing salts, vitamins, amino acids, and glucose, but they produce considerable amounts when harvested from media containing malic acid in addition to the above components. *l*-Malic acid is quantitatively converted to lactic acid and CO_2.[1] This activity is markedly decreased in biotin-deficient cells.[1] The conclusion that an adaptive enzyme is formed is further supported by (*a*) loss of the acquired power to ferment malate after subculture for 24 hours in the absence of this substance and (*b*) failure to extract from unadapted cells an enzyme system active toward malate.

The enzyme system can be readily extracted from washed, acetone-dried, adapted cells with dilute phosphate buffer, pH 7.0, and is fully active after extensive dialysis against salt solutions in the absence of phosphate. The extracts catalyze both reactions (1) and (2).

$$l\text{-Malate} = \text{lactate} + CO_2 \tag{1}$$

$$\text{Oxalacetate} = \text{pyruvate} + CO_2 \tag{2}$$

The requirements for full activity are shown in the accompanying table. The decarboxylation of oxalacetate is not affected by either DPN or TPN. The extracts contain lactic dehydrogenase but little or no malic dehydrogenase as tested spectrophotometrically with reduced DPN and pyruvate or oxalacetate respectively.

Although the ratio of activities (1) and (2) remains constant following partial purification, lactic dehydrogenase is decreased. This suggests that reaction (1) is a DPN-linked dismutation resulting from the combination of reactions (3) and (4).

$$l\text{-Malate} + DPN_{ox.} = \text{pyruvate} + CO_2 + DPN_{red.} \text{ (``malic'' enzyme)} \tag{3}$$

$$\text{Pyruvate} + DPN_{red.} = \text{lactate} + DPN_{ox.} \text{ (lactic dehydrogenase)} \tag{4}$$

The new adaptive enzyme, catalyzing reaction (3), appears to be similar

---

* Aided by grants from the United States Public Health Service, the American Cancer Society (recommended by the Committee on Growth of the National Research Council), the Office of Naval Research, and the Lederle Laboratories Division, American Cyanamid Company. The help of Mr. I. Harary and Miss A. del Campillo is gratefully acknowledged.

[1] Blanchard, M. L., Korkes, S., and Ochoa, S., unpublished.

to the "malic" enzyme of pigeon liver,[2] although strictly DPN- (instead of TPN-) specific. This coenzyme specificity explains the ready occurrence of reaction (1).[3]

Additions	CO$_2$ evolved in 10 min. from	
	0.04 M *l*-malate (pH 6.0)	0.03 M oxalacetate (pH 4.5)
	c.mm.	*c.mm.*
Complete system*.	146	
No phosphate	110	156
" MnCl$_2$	16	12
" DPN	20	165
TPN instead of DPN	25	

* 0.15 M acetate buffer, 0.07 M phosphate buffer, 1.85×10^{-3} M MnCl$_2$ (MgCl$_2$ is much less effective), 1.1×10^{-4} M DPN, and 0.5 cc. of dialyzed enzyme extract containing 4.5 mg. of protein. Final volume, 2.7 cc.; gas, air; temperature, 25°.

Acetone powder extracts of adapted *L. arabinosus* can be conveniently used for the quantitative determination of *l*-malic acid.

Department of Pharmacology
New York University College of Medicine
New York

SEYMOUR KORKES
SEVERO OCHOA

Received for publication, September 10, 1948

---

[2] Ochoa, S., Mehler, A. H., and Kornberg, A., *J. Biol. Chem.*, **174**, 979 (1948).

[3] Mehler, A. H., Kornberg, A., Grisolia, S., and Ochoa, S., *J. Biol. Chem.*, **174**, 961 (1948).

CRYSTALLINE α-AMYLASE FROM BARLEY MALT*

Sirs:

Malted grains are the only rich sources of plant α-amylase that may be obtained in quantity. By the following procedure, we have prepared from barley malt a crystalline protein with a high degree of α-amylolytic activity. The method applies the unusual stability of the crude enzyme toward heat and its highly selective adsorption on raw starch from alcoholic solutions. The instability of the enzyme in the absence of calcium and the advantage of rather high temperatures for crystal formation were found to be important conditions.

A concentrated barley malt extract[1] was heated without dilution to 70° for 10 to 15 minutes and filtered hot. The filtrate was made 0.45 saturated with solid ammonium sulfate, pH being kept at 5.6 to 6.0. The precipitate was washed with about 5 times its volume of one-third saturated ammonium sulfate, made with water saturated with calcium sulfate, and adjusted to pH 5.6 to 6.0. The precipitate was then dissolved in 5 to 10 times its original volume of cold (+5°) dilute ethyl alcohol (40 per cent by volume) containing 2 gm. of $CaCl_2$ per liter. Precipitated calcium sulfate was filtered off in Filter-Cel, and the latter washed with 40 per cent alcohol. The alcoholic solution of amylase was then adsorbed on starch, as outlined by Schwimmer.[2] The starch was washed with cold 40 per cent alcohol and eluted with water saturated with calcium sulfate. It was found best to mix the starch with about an equal weight of Filter-Cel and to suck the alcoholic solution of enzyme through a column of the dry mixture. The eluate was separated into several portions as it came through the column. The portions were tested individually for α-amylase, and only those rich in enzyme were used. The enzyme was precipitated at two-thirds saturation with ammonium sulfate (pH 6.0), removed by filtration with Filter-Cel, washed with more ammonium sulfate solution, and finally extracted from the Filter-Cel with as small a volume as convenient of water half saturated with calcium sulfate.

At this stage, the enzyme solution was clear and colorless, and contained about 2 mg. per cc. of protein nitrogen. The specific activity of the enzyme was about 50 times that of the original malt extract. This solution was brought to pH 5.9 to 6.0 (where α-amylase was found to be least soluble in dilute ammonium sulfate). Crystals appeared on the addition of ammonium sulfate to this solution at about 30°. It was sometimes necessary to remove a fraction precipitated at about 0.2 saturation. The crystalline enzyme separated out, for the most part between 0.25 and 0.28 saturation. Recrystallization was made after solution (which is slow) in half saturated calcium sulfate water and reprecipitation with ammonium sulfate as before. At this point, the specific activities of mother liquor and crystalline material were the same. Crystal formation was not observed below 25°, nor below pH 5.6, though neither condition appeared to affect the activity of the enzyme.

---

* Enzyme Research Laboratory Contribution No. 116.

[1] An experimental product most generously supplied us by Dr. Alexander Frieden of the Pabst Brewing Company, Milwaukee, Wisconsin.

[2] Schwimmer, S., *VII Cong. internat. ind. Agr.*, Paris, 1, Q6-D (1948).

Crystal formation has been observed on many occasions, but the yield was usually small (1 to 2 per cent of the enzyme originally present).

Lately, however, yields of 16 per cent of 2 × crystallized enzyme, and of 11 per cent of 3 × crystallized (both quite free from visible amorphous matter), have been obtained.

The crystals are hexagonal prisms capped by pyramids. They appear to have a high refractive index. The largest obtained were about 13 μ in length. They are readily soluble in 40 per cent alcohol.

Analysis of the twice crystallized material gave 13.4 per cent nitrogen (after dialysis). The crystals did not disintegrate rapidly in 0.4 saturated ammonium sulfate solution that contained no calcium. Crystals washed thoroughly with this solution contained 0.01 per cent P and 0.035 per cent Fe. The ash was 0.64 per cent, of which roughly one-fifth consisted of Ca.[3]

The α-amylolytic activity per unit of protein nitrogen was 67 times that of the original extract. The turnover number is of the same order of magnitude as that of crystalline pancreatic amylase,[4] about 10^5 bonds per molecule per minute (assuming a molecular weight of 100,000). The formation of reducing substances from starch appears to follow the same kinetics as that observed by Bernfeld and Studer-Pécha with partially purified malt α-amylase acting on amylose.[5] This behavior has been regarded as characteristic of α-amylase action.

Enzyme Research Laboratory
Bureau of Agricultural and Industrial Chemistry
United States Department of Agriculture
Albany, California

SIGMUND SCHWIMMER
A. K. BALLS

Received for publication, August 30, 1948

---

[3] Determined spectroscopically through the courtesy of Dr. E. J. Eastmond of the Western Regional Research Laboratory.

[4] Meyer, K. H., Fischer, E. H., and Bernfeld, P., *Helv. chim. acta*, **30**, 64 (1947).

[5] Bernfeld, P., and Studer-Pécha, H., *Helv. chim. acta*, **30**, 1895 (1947).

LIBERATION OF AMINO ACIDS FROM RAW AND HEATED CASEIN BY ACID AND ENZYME HYDROLYSIS*

By L. V. HANKES, W. H. RIESEN, L. M. HENDERSON, and C. A. ELVEHJEM

(*From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*)

(Received for publication, June 19, 1948)

Earlier work showed that the nutritive value of casein was decreased by dry heat and that the addition of lysine restored its nutritive value (1). The lysine was not damaged materially by the heat treatment, since analyses of acid hydrolysates of heated casein with lysine decarboxylase (2) and chemical isolation as the picrate (3) showed no decrease in the lysine content. Eldred and Rodney (2), using the lysine decarboxylase method, and Pader, Melnick, and Oser (4), using the *Streptococcus faecalis* assay, found that heating casein in a dry state at 150° for a few hours decreased the quantity of lysine liberated by enzyme hydrolysis *in vitro*. Block, Jones, and Gersdorff (3) reported that the lysine content of casein was not affected by exposure to dry heat at a temperature at 150°, but that enzymatic liberation of the amino acid was decreased.

Melnick, Oser, and Weiss (5) pointed out that factors known to increase the nutritive value of soy bean protein also increase its *in vitro* digestibility. In a recent report, Riesen *et al.* (6) showed that the degree of liberation of the ten essential amino acids from soy bean oil meal by pancreatin was increased when the meal had been autoclaved for 4 minutes at 15 pounds pressure. When the period of autoclaving was extended to 4 hours, the liberation of these amino acids was decreased below that obtained with the raw meal. The amino acid content was unaffected by the short autoclaving procedure; after prolonged heat treatment, the lysine, arginine, and tryptophan values found by microbiological assay of acid or alkaline hydrolysates were decreased.

To determine whether casein was altered similarly, the effect of heat treatment on the amino acid composition and the extent of liberation of amino acids by enzymes were measured microbiologically. Since preliminary experiments indicated that this protein was much more resistant to changes in digestibility by moist heat than soy bean protein, the longer period of autoclaving was extended from 4 to 20 hours. In this work, the release of amino acids by successive treatment with a number of mammalian digestive enzymes was investigated.

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from Swift and Company, Inc., Chicago, and the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

EXPERIMENTAL

Preparation of Samples—Pyrex trays were filled to a depth of 0.5 inch with vitamin test casein (Smaco) and heated in an autoclave at 15 pounds pressure (121°) for 4 minutes and for 20 hours. The heated casein was then dried in a stream of air at 65° for 24 hours. Moisture and Kjeldahl nitrogen determinations were made on each sample.

Acid and Alkaline Hydrolysis—The conditions of acid hydrolysis which released maximum quantities of amino acids from casein, as measured by the formol titration, were determined in preliminary experiments. Casein was autoclaved with 25 volumes of 3 to 5 N hydrochloric acid at 15 pounds pressure for 1½ to 18 hours; maximum liberation of amino groups was obtained with 3 N acid for 10 hours. Since longer periods of hydrolysis caused a slight reduction of the formol titration value, the 10 hour period with 3 N HCl was adopted for the assay of all amino acids except cystine, tryptophan, and tyrosine.

Riesen (7) found that free cystine was destroyed by the acid hydrolysis procedure used for the release of other amino acids and that maximum cystine values were obtained when the casein was autoclaved with 30 volumes of 2 N hydrochloric acid for 3 hours. This procedure, used in these studies for the hydrolysis for cystine analysis, should give comparable values for the three casein samples, though they may be somewhat lower than the true cystine content.

Alkaline hydrolysates for tryptophan and tyrosine assays were prepared by autoclaving samples of casein with 20 volumes of 5 N sodium hydroxide for 15 hours at 15 pounds pressure. Complete racemization was assumed.

Enzyme Hydrolysis—In enzyme digestion studies, commercial preparations of pancreatic and ereptic enzymes from several sources were assayed for their relative proteolytic or peptidase activities by measuring with formol titration the amino groups liberated from unheated casein. For proteinase activity determinations, 1 gm. of casein was shaken at 37° for 4 hours with 20 mg. of the preparation to be tested at pH 8 with 50 ml. of carbonate buffer. The substrate for peptidase activity determination was prepared by digesting casein for 2 days in this manner with the most active pancreatic enzyme preparation tested. For these assays, 20 mg. of the crude peptidase preparation were incubated at pH 7 for 4 hours with the pancreatic digest of 1 gm. of casein. Pepsin (Difco), whole pancreas (Viobin), and erepsin (Difco) were selected for this work. By employing these enzymes successively for short periods of incubation, the rates of the digestion of the raw and heated casein samples were determined with periodic α-amino nitrogen and microbiological amino acid determinations.

10 gm. each of the raw and the two heated casein samples were placed in 2 liter Erlenmeyer flasks with 500 ml. of 0.1 N hydrochloric acid and shaken at 37° overnight. 10 ml. of enzyme solution containing 50 mg. of pepsin (Difco) were then added to each flask. A fourth flask containing 500 ml. of 0.1 N hydrochloric acid and 50 mg. of pepsin, but no substrate, served as a blank. After 40, 70, and 100 minutes, pH measurements were made and 2 ml. aliquots were removed from each flask for Van Slyke α-amino nitrogen determinations, to measure the progress of the digestion. At 100 minutes, the rate of digestion was decreasing rapidly; therefore, at 120 minutes a 50 ml. aliquot was removed from each flask. These aliquots were heated in a boiling water bath for 15 minutes to inactivate the pepsin, and were stored at −4° for amino acid assays.

Immediately after removal of the 50 ml. aliquots, 8 ml. of 5 N sodium hydroxide were added to each digestion flask to neutralize the solutions partially and to arrest the peptic activity. The solutions were then adjusted to pH 8.2 with 5 N sodium hydroxide and 10 ml. of toluene added. 10 ml. of pancreas (Viobin) solution (filtered water extract containing 10 mg. per ml.) were then pipetted into each flask. The course of the digestion was again followed with pH measurements and Van Slyke α-amino nitrogen determinations; after 1 hour the solutions were readjusted to pH 8.2. After 2 hours 50 ml. aliquots were removed and treated as before.

The contents of each flask were then adjusted to pH 7.0 and 10.0 ml. of erepsin (Difco, filtered water extract containing 5 mg. per ml.) were immediately added. The progress of digestion was again determined by periodic pH measurements and α-amino nitrogen determinations. After 2 hours, 50 ml. aliquots were removed and treated as above. The digestion was allowed to continue for 5 days longer, at which time 50 ml. aliquots were again removed.

Amino Acid Assays—Sixteen amino acids were determined microbiologically on the acid and enzyme hydrolysates with the following organisms for the amino acids indicated: *Lactobacillus arabinosus* 17-5 for glutamic acid, leucine, tryptophan, valine, and phenylalanine; *Leuconostoc mesenteroides* P-60 for aspartic acid, cystine, glycine, histidine, isoleucine, lysine, proline, and tyrosine; *Streptococcus faecalis* R for methionine and threonine; and *Lactobacillus delbrueckii* 3 for arginine. All amino acids except cystine were determined by the methods of Henderson and Snell (8). Cystine was determined with an oxidized peptone medium as described by Riesen *et al.* (9).

RESULTS AND DISCUSSION

Amino Acid Content of Raw versus Heated Casein—The data presented in Table I show that, with the exception of cystine, the amino acid content of casein as measured microbiologically after acid hydrolysis (alkaline hydroly-

Table I

*Liberation of Microbiologically Available Amino Acids from Raw and Heated Casein by Acid and Enzymes**

Amino acid	Amino acid content of acid hydrolysates†				Percentage liberation by digestive enzymes‡											
					Pepsin, 2 hrs			Pepsin + pancreas, 2 hrs. each			Pepsin + pancreas + erepsin, hrs each			Same, continued 5 days		
	Raw	4 min.§	20 hrs §	Average	Raw	4 min §	20 hrs.§	Raw	4 min §	20 hrs.§	Raw	4 min §	20 hrs.§	Raw	4 min.§	20 hrs.§
	per cent	*per cent*	*per cent*	*per cent*												
Arginine	3.20	3.18	3.14	3.17	40	46	28	81	81	58	96	98	72	97	107	89
Aspartic	6.54	6.34	6.17	6.35	1	1	6	1	16	6	5	4	7	11	12	6
Cystine‖	0.50	0.52	0.11	0.51‖	0	1	2	1	3	7	10	7	21	60	40	57
Glutamic	19.27	19.07	18.95	19.10	5	5	3	22	24	17	51	52	36	106	116	83
Glycine	1.69	1.56	1.71	1.65	4	0	4	9	9	9	20	15	16	56	113	48
Histidine	2.60	2.54	2.49	2.54	0	0	1	2	2	5	7	8	8	37	47	34
Isoleucine	5.34	5.61	5.42	5.46	0	0	1	3	3	5	8	8	7	40	49	29
Leucine	8.44	8.61	8.81	8.62	11	9	5	41	44	34	57	58	39	87	99	78
Lysine	6.54	6.59	6.33	6.49	1	0	1	10	10	7	35	33	20	81	81	44
Methionine	2.13	2.11	2.26	2.17	4	4	13	55	59	50	85	90	49	146	150	94
Phenylalanine	4.70	4.49	4.65	4.61	10	13	4	46	48	48	63	64	48	86	119	77
Proline	10.69	9.56	9.34	9.86	0	0	1	1	1	1	1	1	2	19	22	17
Threonine	3.58	3.69	3.50	3.59	3	3	6	27	28	19	44	44	26	83	88	48
Tryptophan¶	1.03	1.09	1.03	1.05	10	12	9	56	64	50	82	87	62	97	100	85
Tyrosine¶	4.51	4.19	4.38	4.36	1	1	1	36	44	31	57	59	35	82	81	77
Valine	6.72	6.62	6.68	6.67	2	2	3	18	20	16	41	49	31	113	132	86
α Amino N**	73††	76††	77††		9	11	10	19	21	17	27	30	24	50	52	40
Amino acid totals‡‡	87.48	85.77	85.00	86.20	5	5	5	22	24	19	39	40	27	77	85	59

* The amino acid figures represent the average of from three to five microbiological assays of two different hydrolysates. The total nitrogen in the raw casein was 13.51, 4 minute casein 13.66, and the 20 hour casein 13.50 per cent; the moisture contents were 8.00, 8.14, and 6.50 per cent, respectively.

† See the text for the conditions of hydrolysis. The acid hydrolysis values are expressed as per cent (gm. per 100 gm. of protein) amino acid yielded by casein; the values for the heated caseins were adjusted to the nitrogen content of the raw casein.

‡ See the text for the conditions of enzyme hydrolysis. The amino acid values were computed by dividing the amount of amino acid liberated by enzymatic hydrolysis by the amount liberated from casein by acid hydrolysis (average of raw and heated caseins) and multiplying by 100. The enzyme α-amino nitrogen figures were computed similarly.

§ The casein was heated by autoclaving for 4 minutes or 20 hours at 15 pounds.

‖ The acid hydrolysis average figure was computed from the liberation from the raw and 4 minute heated casein. Enzymatic liberation values for 20 hour heated casein were computed with the acid liberation figure for this casein sample instead of the average figure.

¶ Since alkaline hydrolysis was used, complete racemization was assumed and figures represent twice the actual amounts measured.

** Determined by the semimicro nitrous acid method of Van Slyke.

†† The figures represent the percentage of the total nitrogen released in the Van Slyke procedure.

‡‡ Acid hydrolysis totals are the sums of the percentages of the amino acids liberated. Enzyme hydrolysis totals were obtained by dividing the total amount of microbiologically available amino acids liberated enzymatically by the total amount liberated from the corresponding casein sample by acid hydrolysis (or alkaline hydrolysis) and multiplying by 100.

sis for tyrosine and tryptophan) was not affected significantly by autoclaving at 15 pounds pressure (121°) for 4 minutes or 20 hours. The cystine value was reduced to one-fourth that of the raw casein by autoclaving for 20 hours, but was unchanged by autoclaving for 4 minutes.

Liberation of Amino Acids by Digestive Enzymes—In Fig. 1 are shown typical hydrolysis curves of raw casein and casein autoclaved for 4 minutes and 20 hours at 15 pounds when subjected to successive digestion with pepsin, pancreas enzymes, and erepsin. Heated casein was digested more rapidly in the initial stages by pepsin; the extent of digestion was about the same at the end of 2 hours, regardless of the heat treatment. The rate of release of α-amino nitrogen during pancreatic and ereptic digestion of casein was slightly increased by autoclaving for 4 minutes and decreased by autoclaving for 20 hours. A disproportionately large reduction in pH in relation to the release of α-amino nitrogen occurred during the 1st hour of pancreatic digestion.

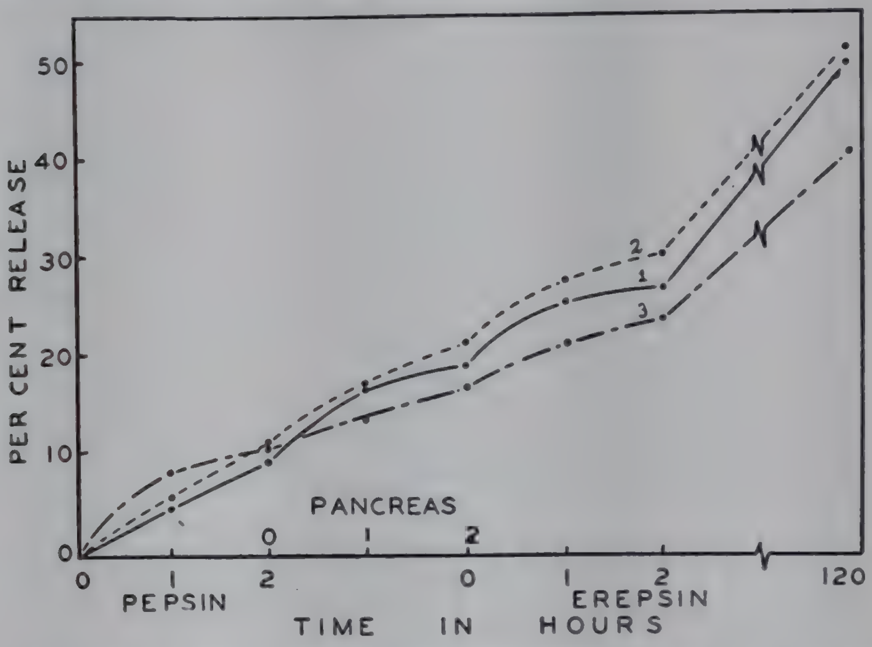


FIG. 1. Release of α-amino nitrogen from raw casein (Curve 1), casein autoclaved 4 minutes (Curve 2), and casein autoclaved 20 hours (Curve 3) by successive digestion with pepsin, pancreas enzymes, and erepsin. The digests were adjusted to pH 1.0 for peptic digestion, to pH 8.0 for pancreatic digestion, and to pH 6.8 for ereptic digestion. There were insignificant changes in pH during the course of the digestion, except after 1 hour with pancreas enzymes when the raw casein digest was pH 6.4, 4 minute casein pH 5.9, and the 20 hour casein pH 7.1. The contents were readjusted to pH 8 for the remainder of the pancreatic digestion.

The interpretation of the data on the extent of amino acid liberation by enzymes when measured by microbiological procedures is complicated by the probable utilization of peptides by the microorganisms commonly used for the assay of amino acids. Peptides that have been investigated thus far show variable activity, ranging from 0 to 100 per cent when assayed for the amino acids which they contain (10–13). In view of this variation in response of microorganisms to peptides, the term "microbiologically available" amino acids will be used in the discussion of Table I. The above objection invalidates these values for other than gross comparative purposes.

Pepsin Digestion

The liberation of α-amino nitrogen and microbiologically available amino acids from casein by pepsin was unaffected by heat treatment. There was considerable variation in the extent of liberation of the individual amino acids; a relatively large percentage of the arginine and much lower percentages of glutamic acid, glycine, leucine, methionine, phenylalanine, threonine, and tryptophan were released. No significant quantities of the other amino acids were liberated. The percentage of arginine that became microbiologically available was about 8 times as great as the average[1] percentage of all sixteen amino acids. Half of the α-amino nitrogen liberated (measured by the Van Slyke method) could be accounted for by microbiologically available amino acids.

Pepsin Plus Pancreas Digestion

The liberation of α-amino nitrogen and the "average" liberation of amino acids from casein by pepsin followed by pancreas were slightly increased by autoclaving for 4 minutes and decreased by autoclaving for 20 hours. All amino acids except proline were released by pancreas enzymes. The extent of liberation of aspartic acid, cystine, histidine, and isoleucine was less than 10 per cent, while the total amino acid liberation was approximately 20 per cent and was equal to the percentage release of α-amino nitrogen.

The percentage of the arginine which became microbiologically available was approximately 4 times as great as the average of the other amino acids. Hunter and Dauphinee (14) have also reported a rapid cleavage of this amino acid from casein and gelatin by trypsin. It is not known, however, whether arginine is liberated in the form of peptides having high activity for *Lactobacillus delbrueckii* 3 or as free arginine.

In general the results obtained with pancreas enzymes agree with those of other workers. Abderhalden (15) found more rapid liberation of tyrosine than of glutamic acid from casein by pancreatin. Hunter (16) found that a proline fraction exists in casein which is comparatively resistant to tryptic digestion. It is possible that the low values obtained in the present study with enzyme digests are due to the absence of proline-releasing enzymes in the preparations used.

---

[1] See Table I, foot-note ‡‡.

Digestion with Pepsin, Pancreas, and Erepsin

The extent of liberation of microbiologically available amino acids from casein was determined after following the pepsin-pancreatic digests prepared in the above manner with digestion by erepsin for 2 hours and 5 days. The extent of liberation of aspartic acid, cystine, histidine, isoleucine, and proline after 2 hours was below 10 per cent, while the total liberation of amino acids was about 40 per cent. Arginine, methionine, and tryptophan were nearly entirely liberated. After 5 days, most amino acids were completely available to the microorganisms used for assay. The liberation of cystine, which had been low throughout the digestion, increased considerably after 5 days. The release of aspartic acid and proline remained low, *i.e.* 10 to 20 per cent, while the liberation of methionine was approximately 150 per cent. The latter result could indicate activity of peptides above that expected on the basis of methionine content, or destruction of methionine during acid hydrolysis. The values for acid hydrolysates reported here are lower than those obtained by many other workers, a result which supports the latter explanation. In a previous study, an average value of 2.69 ± 0.26 per cent in the dried, ash-free protein was reported, while six values cited from the literature averaged 2.85 ± 0.21. The value for raw casein reported in Table I is 2.33 corrected for moisture and ash.

The amino acid totals exceeded the α-amino nitrogen values after digestion by erepsin for 2 hours or for 5 days. The average peptide size at the end of 5 days was 2 amino acid residues.

It should be pointed out that the extent of liberation of glutamic acid from proteins by enzymes is not strictly comparable to that obtained on acid hydrolysates, since in enzyme hydrolysates any glutamine released would have been converted to pyrrolidonecarboxylic acid by the heating to inactivate the enzymes, and the subsequent sterilization of the assay tubes by autoclaving. According to Hamilton (17) glutamic acid is relatively stable to acid hydrolysis and to autoclaving, whereas glutamine is converted to glutamic acid when heated below pH 3 and to the inactive pyrrolidonecarboxylic acid during autoclaving at pH 6.5. Work in this laboratory has shown that sterilization of the medium by autoclaving for 10 minutes at 12 pounds pressure at neutral pH causes complete or nearly complete loss of activity of free glutamine for *Lactobacillus arabinosus*. It appears from Table I that glutamic acid was quantitatively liberated by enzymes in 5 days or less. This may be accounted for by assuming (*a*) deamidation of the glutamine during enzyme hydrolysis, (*b*) release of glutamine in peptide combinations which are not cyclized by heat, or (*c*) release of peptides of glutamic acid possessing more activity than glutamic acid itself, thus compensating for the glutamine destroyed during autoclaving.

The liberation of aspartic acid, in contrast to glutamic acid, was low throughout the 5 day digestion period. Whereas glutamine is fully as active as glutamic acid for *Lactobacillus arabinosus* (18), asparagine is much less active than aspartic acid for *Leuconostoc mesenteroides* P-60 (19). The low values obtained may be due to the liberation in the form of asparagine or peptides.

Casein appears to be less affected by heat than soy bean protein. Very little increase in digestibility was noted after 4 minutes of autoclaving; some destructive effects occurred after 20 hours. In both soy bean and casein, lysine was among the amino acids whose rate of release by enzymes after heat treatment was affected most adversely.

SUMMARY

1. Autoclaving casein at 15 pounds pressure for 4 minutes had no effect on the amino acid composition as measured by microbiological determinations after acid hydrolysis. Autoclaving for 20 hours reduced the cystine content, but did not affect the amounts of other amino acids.

2. The rate of release of α-amino nitrogen and of microbiologically available amino acids during a 2 hour digestion of casein with a limited quantity of pepsin was unaffected by the heat treatments. The release of amino acids from the pepsin digests after treatment with desiccated pancreas and then with erepsin was higher in the casein autoclaved for 4 minutes and lower in casein autoclaved for 20 hours, compared to unheated casein.

3. The release of amino acids from raw casein by pepsin was 5 per cent with 10 per cent liberation of α-amino nitrogen. 40 per cent of the arginine became microbiologically available.

4. Pancreatic digestion for 2 hours released approximately 22 per cent of the amino acids and 19 per cent of the α-amino nitrogen and, after an additional 2 hour digestion with erepsin, 39 per cent of the amino acids was available to the lactic acid bacteria and 27 per cent of the α-amino nitrogen was released. Continued digestion with no additional enzymes for 5 days released 50 per cent of the α-amino nitrogen and 77 per cent of the amino acids in microbiologically available form. Only small amounts of aspartic acid and proline were liberated.

BIBLIOGRAPHY

1. Greaves, E. O., Morgan, A. F., and Loveen, M. N., *J. Nutr.*, **16**, 115 (1938).
2. Eldred, N. R., and Rodney, G., *J. Biol. Chem.*, **162**, 261 (1946).

3. Block, R. J., Jones, D. B., and Gersdorff, C. E. F., *J. Biol. Chem.*, **105**, 667 (1934).
4. Pader, M., Melnick, D., and Oser, B. L., *J. Biol. Chem.*, **172**, 763 (1948).
5. Melnick, D., Oser, B. L., and Weiss, S., *Science*, **103**, 326 (1946).
6. Riesen, W. H., Clandinin, D. R., Elvehjem, C. A., and Cravens, W. W., *J. Biol. Chem.*, **167**, 143 (1947).
7. Riesen, W. H., Thesis, University of Wisconsin (1947).
8. Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172**, 15 (1948).
9. Riesen, W. H., Spengler, H. H., Robblee, A. R., Hankes, L. V., and Elvehjem, C. A., *J. Biol. Chem.*, **171**, 731 (1947).
10. Schweigert, B. S., and Snell, E. E., *Nutr. Abstr. and Rev.*, **16**, 497 (1946–47).
11. Ågren, G., *Acta physiol. Scand.*, **13**, 347 (1947).
12. Hegsted, D. M., *J. Biol. Chem.*, **157**, 741 (1945).
13. Krehl, W. A., and Fruton, J. S., *J. Biol. Chem.*, **173**, 479 (1948).
14. Hunter, A., and Dauphinee, J. A., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **63**, p. xxxix (1925).
15. Abderhalden, E., Lehrbuch der physiologischen Chemie, Berlin, 5th edition, **1**, 468 (1923).
16. Hunter, A., *Tr. Roy. Soc. Canada*, **16**, 71 (1922).
17. Hamilton, P. B., *J. Biol. Chem.*, **158**, 375 (1945).
18. Hac, L. R., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **159**, 273 (1945).
19. Hac, L. R., and Snell, E. E., *J. Biol. Chem.*, **159**, 291 (1945).

THE DISTRIBUTION OF COLLAGEN IN THE GUINEA PIG

By LOIS H. LIGHTFOOT and THOMAS B. COOLIDGE

(*From the Department of Biochemistry and the Walter G. Zoller Memorial Dental Clinic, University of Chicago, Chicago*)

(Received for publication, June 19, 1948)

Collagen is an extracellular protein found in connective tissue, especially in bone, skin, tendon, fascia, and the walls of blood vessels. Similarities in respect to biological origin, staining reactions, solubility in alkali, production of gelatin on boiling, etc., appear to justify the assumption generally made that collagen is of the same general nature in all the mammalian tissues in which it occurs. It has been shown histologically by Wolbach and Howe (1) that collagen is formed from precollagenous material in maturing connective tissue and that this process is interrupted in scurvy.

The occurrence of collagen in tissues whose primarily mechanical function is extended geometrically in growth and maintained in fasting has made it seem desirable to investigate (1) the distribution of collagen in the whole animal and in individual tissues, and (2) the variations in collagen distribution in fasting and scorbutic animals at different age levels.

In the following study the collagen method of Spencer, Morgulis, and Wilder (2) (which they tested with some care for specificity, and which in our hands has yielded more reproducible results with small amounts of tissue than did the method of Lowry, Gilligan, and Katersky (3)) has been modified to render it applicable to bone, and the precision of the modified method has been determined. The collagen nitrogen, expressed as per cent of total nitrogen, has been determined in the muscle, connective tissue, and bone of normal, scorbutic, and fasted guinea pigs at three age levels. An effect of scurvy on this percentage is demonstrable in the bones of young animals, the effect being consistent with the histological findings mentioned above (Experiment 1).

Because the increase in the amount of collagen relative to total nitrogen in muscle in fasting found with the method of Spencer, Morgulis, and Wilder appeared unexpectedly high, the increase was measured by the method of Lowry, Gilligan, and Katersky (Experiment 2). The latter method was also employed in an approximate analysis of the distribution of collagen in a whole guinea pig (Experiment 3).

EXPERIMENTAL

Experiment 1

Methods—The experimental animals consisted of three sets of six guinea pigs each. The animals of the first set were 10 days old at the beginning of the experiment, those of the second set 36 days old, and those of the third set were adult (at least 72 days old). Of each set, two animals were fed a complete diet, two a vitamin C-free diet, and two were given a complete diet for 10 to 12 days from the start of the experiment, the procedure being designed to bring the fasted animals to about the same weight as the scorbutic animals at the end of the experiment. All six animals in each set were killed at approximately the same time. All the animals on a scorbutic diet manifested losses of weight and (with the exception of Animal 17) showed the gross appearance and behavior of scorbutic animals, and all showed gross lesions characteristic of scurvy in either mandible or tibia. The adequate diet consisted of cabbage, carrots, hay, and either Purina rabbit chow or vitamin C-free rabbit pellets (Arcady Farms Milling Company, Chicago); the scorbutic diet consisted of vitamin C-free rabbit pellets.

From each of the freshly killed animals were removed (1) one gastrocnemius muscle, *which was rapidly freed of its tendons and grossly visible fascia,* (2) transparent pieces of fascia about 0.2 sq. cm. in area and free of visible vessels, nerves, and fat from beneath the dorsal skin, about eight such pieces being pooled in a centrifuge tube containing absolute alcohol and two or three such pooled samples being taken per animal, and (3) the femurs.

Each muscle was immediately ground with a little water to a homogeneous suspension; the suspension was diluted with water to about 20 ml., and four 4.00 ml. aliquots were pipetted into 15 ml. centrifuge tubes which were immediately heated in the autoclave at 21 pounds pressure for 3 hours, cooled, and centrifuged. Each supernatant with two 2.0 ml. washings of the corresponding precipitate was transferred to a second 15 ml. centrifuge tube and mixed with 6 drops of concentrated HCl and 4.0 ml. of 5 per cent tannic acid. After standing overnight at 4° the precipitate was centrifuged and the supernatant and two 2.0 ml. washings of the precipitate were pooled and analyzed for nitrogen (acid-soluble non-collagen nitrogen). The washed precipitate from the autoclaving and the washed precipitate from the tannic acid precipitation were analyzed, yielding acid-insoluble non-collagen nitrogen and collagen nitrogen respectively.

Each pooled sample of fascia, after removal of the ethanol and suspension in 4.0 ml. of water, was analyzed in similar fashion, the ethanol being added to the acid-soluble non-collagen nitrogen fraction.

The femurs were carefully freed of muscle and tendon, left in acetone overnight, and dried at 110° for 30 minutes. Each bone was ground until the particles passed through a 200 mesh sieve. Accurately weighed samples about 50 mg. in weight were placed in 15 ml. centrifuge tubes with 4 ml. of water and autoclaved for 3 hours at 30 pounds pressure. The tubes were cooled and centrifuged and the supernatants poured into 15 ml. centrifuge tubes, diluted to about 8 ml., and treated with 6 drops of concentrated HCl and 4 ml. of 5 per cent tannic acid as above. The precipitates separated from the above supernatants were shaken an hour with 8 ml. of 0.5 N HCl, centrifuged, and retreated with 0.5 N HCl. The supernatants from both treatments with 0.5 N HCl were joined and precipitated with 8 ml. of 5 per cent tannic acid. The tannic acid precipitates, the supernatants overlying them after centrifugation, and the material not rendered soluble by HCl were treated as were the similar fractions from muscle. Other aliquots of the bones were analyzed for total nitrogen.

The method outlined is a micromodification of the method of Spencer, Morgulis, and Wilder (2). The digestion in the nitrogen analyses was begun with 2.0 ml. (4.0 ml. for non-collagen nitrogen) of concentrated sulfuric acid in a total volume of at least 6 ml. and continued for 12 hours with selenized Hengar granules. The distillation and titration were carried out according to Ma and Zuazaga (4). The average total nitrogen in the muscle aliquots was about 10 mg. per aliquot, in the pooled fascia samples 0.2 mg. per pooled sample, and in the bone aliquots 2 mg. per aliquot.

Results—Table I includes all results not discarded for known errors in technique, except that the separate figures for acid-soluble and acid-insoluble non-collagen nitrogen and bone total nitrogen are not included. The average deviation from the mean is about 0.1 mg. of nitrogen in the muscle analyses, 0.03 mg. of N in the bone analyses, and 0.01 mg. of N in the fascia analyses. The percentage of total nitrogen in the acid-soluble fraction was relatively constant in all three tissues. Deviations from the mean values of 20 per cent for muscle, 24 per cent for fascia, and 14 per cent for bone, except for slightly higher values in the youngest animals, lie within the range of experimental error. In all three tissues, therefore, the collagen nitrogen and acid-insoluble non-collagen nitrogen constitute about 80 to 85 per cent of the total nitrogen. (For example, the figures for muscle collagen nitrogen and acid-insoluble non-collagen nitrogen are 10 and 70 per cent respectively for Animal 5, and 50 and 30 per cent respectively for Animal 11.)

The sum of the nitrogen in the bone fractions agreed with the total nitrogen determined independently, with an average deviation of 3 per cent and no deviation over 8 per cent. An exception was the analysis of Animal 13, in which a 20 per cent deviation is attributable to an error in the total nitrogen analysis, which explains the low collagen nitrogen expressed as per cent of dry bone weight when compared with the analysis of Animal 13.

In Table I the drop from the normal values of bone collagen nitrogen in scurvy is marked in the youngest animal and appreciable in the middle age group. As there is no effect of fasting in these groups, the effect of

Table I

Variations in Collagen Distribution (Experiment 1)

Animal No.	Age	Diet		Weight during experiment		Collagen N, per cent of total N			Collagen N, per cent of dry bone weight
		Nature	Duration	Initial	Final	Muscle	Fascia	Bone	
	days		*days*	*gm.*	*gm.*				
1	10	Adequate	22	131	277	10	76	51	2.35
						10	61		
						10	58		
						14			
2	10	"	22	138	286	9	61	54	2.50
						9	37	48	
						10			
						10			
3	37	"	25	302	472	13	66	64	2.52
						12	60	61	
						12	70		
						11			
4	37	"	25	326	502	12	74	60	2.64
						14	62	60	
						12	71		
						14			
5	90	"	29	466	643	11	72	70	2.62
						11	61		
						9	54		
						10			
6	90	"	29	459	631	10	50	68	2.58
						10	56	66	
						10	67		
						7			
7*	10	"	15	116	120	55	57	52	2.65
		No food	7			53	48	48	
						53	58		
						53			
8	10	Adequate	15	127	152	57	39	57.5	3.16
		No food	7			58	40	57	
						56	48		
						57			
9	37	Adequate	16	302	256	55	67	66	2.71
		No food	9			73			
						66			
						67			
10*	37	Adequate	16	326	262	60		68	2.90
		No food	9			54		67	
						59			
						53			

TABLE I—*Concluded*

Animal No.	Age	Diet		Weight during experiment		Collagen N, per cent of total N			Collagen N, per cent of dry bone weight
		Nature	Duration	Initial	Final	Muscle	Fascia	Bone	
	days		*days*	*gm.*	*gm.*				
11*	90	Adequate	11	523	365	41	64	58	2.38
		No food	11			51	84	57	
						51			
						49			
12	90	Adequate	11	679	465	13	68	62	2.20
		No food	11			19	78	60	
						14	75		
						14			
13	10	Scorbutic	22	108	146	16	62	37	1.77
						17	66	32	
						13			
13†								33	2.01
								33	
14	10	"	22	108	146	20	57		
						22	92		
						24	62		
						24			
15	37	"	25	348	365	22	35	54	2.43
						20	62	52	
						19	73		
						13			
16	37	"	25	343	334	13	56	50	2.35
						15	62		
						18			
						18			
17	90	"	29	598	577	16	72	73	2.56
						18	72	74	
						19			
						15			
18	90	"	29	610	420	11	68	68	2.68
						10	79	67	
						13	64		
						11			

* Died.

† Left femur of Animal 13.

scurvy is not to be ascribed to inanition. The drop is consistent with the histological findings in scorbutic bone, an interruption of the conversion of precollagenous material to collagen. An increase in the collagen per cent with age and a decreased effect of scurvy with age are observable by an inspection of the data and are statistically significant. The constancy of the collagen nitrogen expressed as per cent of dry bone weight in normal, fasted, and older scorbutic animals is to be noted.

In muscle the direction (rise) of the changes in the collagen nitrogen is the same in both fasting and scurvy and the rise is greater in fasting. Hence the rise in scurvy may be attributed to inanition.

The fascia analyses are obviously at the lower limit of useful precision of the method in its present form. The average collagen values for normal, fasting, and scorbutic animals are 62, 61, and 66 per cent respectively; for the youngest, intermediate, and oldest animals, 58, 64, and 70 per cent respectively, the probable error being about 2 per cent for each average. There is a significant increase in collagen per cent with age. It is demonstrably improbable that scurvy produces a considerable (5 per cent) decrease in collagen percentage at all ages.

TABLE II

Collagen and Total Nitrogen Content of Gastrocnemius (Experiment 2)

Animal No.	Diet		Weight during experiment		Gastrocnemii, right and left combined				
	Nature	Duration	Initial	Final	Weight	Collagen N	Total N	$\frac{\text{Collagen N}}{\text{Total N}} \times 100$	$\frac{\text{Collagen N}}{\text{Animal weight}}$
		days	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*		*mg. per kg.*
A	Water	8	440	310	2.30	12.0	77.1	15.6	38.7
B	"	9	430	240	1.30	11.4	45.7	24.9	47.5
C	Adequate	9	400	440	2.95	11.1	98.3	11.3	25.2
D	"	9	400	480	2.69	11.2	90.6	12.4	23.3
E	"	0		410	2.42	8.65	75.4	11.5	21.1
F	"	0		380	2.38	8.62	75.9	11.4	22.7

Experiment 2

Six growing guinea pigs were used. Four were of approximately equal weight at the start of the experiment and, of these, two were continued on a normal diet and two received water but no food from the start of the experiment. One of the latter died on the 8th day of the experiment and the remaining three, together with two animals having approximately the same weight that the original four had at the start of the experiment, were killed at the same time, 9 days after the start. From each animal both gastrocnemius muscles *with tendons and investing fascia* were removed and weighed. Collagen and total nitrogen were determined by the general method of Lowry, Gilligan, and Katersky, except that nitrogen analyses replaced the weighings. The results are given in Table II.

Experiment 3

A 420 gm. guinea pig was rapidly dissected into four fractions: skin, large muscles and fascia, bones and adherent muscles and fascia, and organs. The four fractions were weighed and analyzed as in Experiment 2, except that the "bone and adherent muscle and fascia" fraction was not ground fine but extracted for 3 days with 0.1 N NaOH (instead of the usual fine grinding and 14 hour extraction). At the end of 3 days the tissue was almost completely colorless. Neutralization and autoclaving of this fraction were completed and the undissolved bone and fiber (dry weight, 14.5 gm.) were analyzed separately for total nitrogen (188 mg.), this nitrogen being assigned arbitrarily half to the collagen and half to the non-collagen nitrogen of the "bone and adherent muscle and fascia" fraction. Shaved hair from the skin, the feces, and the blood shed during the dissection were collected separately, and the nitrogen content of the blood was calculated. All nitrogen analyses were done in duplicate and the figures in Table III are subject to errors of not greater than 2 or 3 per cent arising from variation in the nitrogen analyses.

TABLE III

Distribution of Collagen (Experiment 3)

	Weight	Collagen N	Total N
	gm.	*mg.*	*mg.*
Whole animal	420		
Skin, shaved	61	1243	3361
Large muscles, tendon, and fascia	106	270	669
Bones, adherent muscles, and fascia	78	942	2359
Organs	80	88	1507
Hair	15		
Feces	42		
Shed blood	35		1000*
Hairless animal	360	2543	8896

Total collagen nitrogen = 29 per cent of total nitrogen.

* Estimated.

DISCUSSION

The analyses summarized in Table II show that in the fasted animal at the growing age collagen continues to be synthesized at about the same rate as in the well fed animal. The absolute values for collagen nitrogen estimated by the different methods employed in Experiments 1 and 2 are not more than approximately comparable (3), and the 2-fold increase in collagen nitrogen during fasting, expressed as per cent of total nitrogen observed in Animal B, Table II, represents the relative increase in collagen in a whole muscle including tendon and investing fascia, while the 4-fold increases during starvation in Table I occur in samples from which tendon and fascia have been removed. The results by the two methods are not therefore necessarily in disagreement.

The analysis summarized in Table III shows that the fascia contains a large fraction of the total collagen of the guinea pig, and the large amount of fascia in the thoracic wall was made strikingly apparent when the crudely dissected skeleton was stirred with 0.1 N NaOH. The analysis of the whole animal has not the precision attained with smaller samples which are more carefully ground, but it clearly demonstrates that collagen formation is quantitatively the most important single reaction leading to the sequestration of nitrogen for growth.

Collagen is localized in tissues which are primarily concerned with the maintenance of the body's gross form against mechanical pressures arising within the body (*e.g.*, muscle tonus, blood pressure) and balanced partially by forces in the environment (*e.g.*, the pressure of gravity on the muscles, tendons, and bones; the atmospheric pressure). The growth of such structures is a geometrical extension of the tissues against the pressures exerted upon them by the environment and is a characteristically biological evasion of the principle of Le Chatelier. The observation of Wolbach and Howe (1) that blood vessels fail to grow into wounded areas produced in scorbutic guinea pigs may be of interest in this respect.

SUMMARY

A micromethod for the determination of collagen in bone is described.

The distribution of collagen in the guinea pig and the effect of age and fasting on the muscle collagen of guinea pigs has been investigated.

Collagen nitrogen constitutes a considerable fraction of the total body nitrogen, and during growth collagen appears to be synthesized in fasting animals at about the same rate as in non-fasting animals.

We here thank Dr. Friedrich Wassermann and Miss Rebecca Woodson for essential assistance in undertaking and in completing this study.

BIBLIOGRAPHY

1. Wolbach, S. B., and Howe, P., *Arch. Path. and Lab. Med.*, **1**, 1 (1926).
2. Spencer, H. C., Morgulis, S., and Wilder, V. M., *J. Biol. Chem.*, **120**, 257 (1937).
3. Lowry, O. H., Gilligan, D. R., and Katersky, E. M., *J. Biol. Chem.*, **139**, 795 (1941).
4. Ma, T. S., and Zuazaga, G., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 280 (1942).

SYNTHESIS AND PROPERTIES OF TRIACETIC ACID*

By ROBERT F. WITTER† and ELMER STOTZ†

(From the Biochemical Laboratories, Division of Food Science and Technology, New York State Agricultural Experiment Station, Cornell University, Geneva, and the Department of Biochemistry, School of Medicine and Dentistry, The University of Rochester, Rochester, New York)

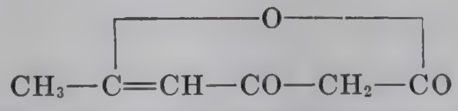
(Received for publication, July 8, 1948)

Advances in intermediary fat metabolism have been restricted considerably by the lack of postulated intermediate compounds. Triacetic acid (β,δ-diketohexanoic acid) would be the product of multiple alternate oxidation of hexanoic acid, and would also be the first product resulting from the condensation of acetoacetic acid with acetate by a mechanism postulated for fatty acid synthesis (1). Triacetic lactone, loosely referred to as "triacetic acid" by Breusch and Ulusoy (2, 3), has been used in metabolic experiments by these investigators, but to the authors' knowledge free triacetic acid has not been isolated or tested for biological activity.

The compounds under principal consideration are represented in the accompanying formulas.

$CH_3{-}CO{-}CH_2{-}CO{-}CH_2{-}COOH$

Triacetic acid (β,δ-diketohexanoic acid)



$CH_3{-}C{=}CH{-}CO{-}CH_2{-}CO$ (ring closed through O)

Triacetic lactone (6-methylpyranone)

$CH_3{-}CO{-}CH_2{-}CO{-}CH_2{-}COOC_2H_5$

Ethyl triacetate

$CH_3{-}CO{-}CH_2{-}CO{-}CH_3$

Acetylacetone

In this paper the synthesis of triacetic acid is described, involving the following series of reactions: dehydroacetic acid → triacetic lactone → copper ethyl triacetate → ethyl triacetate → triacetic acid. Triacetic lactone was condensed with ethyl alcohol in a sealed tube to break the stable enol ring, with formation of ethyl triacetate, and the latter was isolated as the stable copper salt by a modification of Sproxton's procedure (4). Isolation of the free acid involved prior study of the conditions of esterification to preserve the unstable acid. Properties of the free acid, including its color reaction with *o*-phenylenediamine and its catalytic decarboxylation with various aromatic amines, are reported. Analytical data on the free acid and its 2,4-dinitrophenylhydrazine derivatives are also given.

---

* Journal Paper No. 750, New York State Agricultural Experiment Station, Cornell University, Geneva, New York. This research was supported by grants from the Nutrition Foundation, Inc., the Sugar Research Foundation, Inc., and the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service.

† Present address, School of Medicine and Dentistry, The University of Rochester.

Methods

In order to determine the proper conditions for hydrolysis of triacetic ester and to compare the properties of triacetic acid with acetoacetic acid, catalytic decarboxylation by aromatic amines was employed. In this method triacetic ester and lactone are not decarboxylated, while the free acid is readily attacked. Decarboxylation methods with aniline citrate (5), aniline acetate-sodium cyanide (6), *o*- and *p*-phenylenediamine citrates (7), as well as modifications of these methods were variously used. From 3 to 8 micromoles of the β-keto acids were employed in a 3 ml. volume in Warburg flasks, and the catalysts added from the side arm after appropriate equilibration of the reaction mixtures at 30°. The flasks were filled with nitrogen when phenylenediamine catalysts were employed.

The *o*-phenylenediamine color reaction described in the following paper (8) was also employed in controlling the isolation of triacetic acid. This reaction is given directly by triacetic acid, triacetic ester, and acetylacetone which contain a β-diketone group ($—CH_2—CO—CH_2—CO—CH_2—$). Triacetic lactone does not react directly but can be converted to acetylacetone by acid hydrolysis.

Solutions of acetoacetic acid used in these studies were prepared according to the method of Krebs and Eggleston (5).

Synthesis of Triacetic Acid

Triacetic Lactone—Triacetic lactone was prepared from dehydroacetic acid (Eastman) according to the method described by Collie (9). The product had a melting point of 186–187° (Collie, 187–188°) and a neutral equivalent of 130 (Collie, 130; theoretical 126).

Copper Ethyl Triacetate—This substance was previously isolated by Sproxton (4). A mixture of 7.56 gm. (0.06 mole) of triacetic lactone and 80 to 90 ml. of absolute ethyl alcohol was heated in a dry sealed tube at 110° for 48 hours. After cooling, the contents were diluted to 100 ml. with ethyl alcohol and analyzed for diketones (8). Analysis showed that 43 per cent of the lactone remained unchanged, and 47 per cent was present as ethyl triacetate, a total of 90 per cent of the original lactone being accounted for. No reaction took place at 100°, and heating for more than 48 hours did not increase the yield.

To the alcohol solution were added 0.5 mole of copper acetate and a slight excess of ammonia for each mole of ethyl triacetate found (in the above case 0.014 mole of copper acetate as a 0.3 M aqueous solution and

0.03 mole of ammonia as concentrated ammonium hydroxide). After 2 hours standing at room temperature the gray-blue precipitate was collected, washed with cold ethyl alcohol, and dried over P_2O_5 *in vacuo*. The yield was 3.3 gm. or 58 per cent of the copper salt calculated to be present in the solution. The melting point was 182.5–183.5°. Sproxton (4) reported 183–184°.

No precipitate formed in the absence of ammonia, the yield was lowered if a greater excess of ammonia was added, and only a small amount of impure product was obtained if the mother liquor was cooled to 5° or concentrated *in vacuo* to a small volume.

Ethyl Triacetate Solution—200 to 400 mg. of copper ethyl triacetate were finely suspended in 5 ml. of water and H_2S passed through the ice-cold solution for 6 hours. The mixture was filtered, diluted to 10 ml., and aerated to remove excess H_2S. Diketone analysis indicated a 97 to 100 per cent yield of ethyl ester.

Isolation of Triacetic Acid—To the 10 ml. of triacetic ester solution (0.1 to 0.2 M) resulting from the H_2S decomposition of copper ethyl triacetate was added 1 N sodium hydroxide in the ratio of 2 moles of alkali for each mole of triacetic ester present. After standing at 5° for 24 hours, the solution was centrifuged and the clear supernatant liquid acidified to Congo red with 1 N sulfuric acid. The solution was then extracted three times with equal volumes of redistilled ethyl ether, the combined ether extracts washed with 0.1 volume of water, dried with anhydrous sodium sulfate for 1 hour, and finally evaporated in a stream of air. The oily residue was placed in a vacuum desiccator over sulfuric acid at 5°. After 3 to 4 days a light yellow, waxy solid, consisting of rhombic plates, appeared. The triacetic acid yield was 40 to 50 per cent of the theoretical calculated from the copper ethyl triacetate used.

After recrystallization from ethyl ether-petroleum ether, triacetic acid had a melting point of 29–31°, a neutral equivalent of 147 (theory for $C_6H_8O_4$, 144), and a pK of approximately 3.3. The acid gave the following analysis.

$C_6H_8O_4$. Calculated, C 50.0, H 5.55; found, C 49.2, H 5.55

The 2,4-dinitrophenylhydrazine derivatives of triacetic acid were prepared by the general method of Clift and Cook (10) as follows: To a 0.04 M solution of triacetic acid was added an equimolar amount of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid. After standing overnight at 5°, the precipitate was dissolved in saturated Na_2CO_3. The light brown solution was acidified at 5° with concentrated HCl, and the yellow derivative which separated melted at 155–182°. Upon solution in ethyl acetate, this material was separated into two components by precipitation with petroleum ether. With 2 volumes of petroleum ether, yellow hexagonal crystals separated which had a melting point of 189–191° (with gas evolution). 4 volumes of petroleum ether precipitated yellow needle-like crystals, with a melting point of 149–150° (with gas evolution). After drying the derivatives *in vacuo* over H_2SO_4, their neutral equivalents were determined by the method of Clift and Cook (10). The method yielded theoretical values with acetoacetic acid, with brom-thymol blue as the indicator. Nitrogen analyses by the micro-Dumas method were also made, with the following results.

$C_{12}H_{12}O_7N_4$.	Calculated.	N 17.28,	neutral	equivalent	324
M.p. 189–191°.	Found.	“ 17.86	“	“	326
“ 149–150°.	“	“ 17.74	“	“	324
$C_{12}H_{10}O_6N_4$.	Calculated.	“ 18.30	“	“	306
M.p. 189–191°.	Found.	“ 17.86	“	“	326
“ 149–150°.	“	“ 17.74	“	“	324

Both derivatives thus had the same neutral equivalent and nitrogen content. Neither gave a Knorr test (11) for the pyrazoline ring after reduction with sodium in ethyl alcohol. The neutral equivalent and negative Knorr test indicate an open chain 2,4-dinitrophenylhydrazone, although the nitrogen analysis falls between the theoretical values for an open chain compound and a ring structure.

Properties of Triacetic Ester

Dilute aqueous solutions (0.02 to 0.1 M) of ethyl triacetate are light yellow in color and have a sweet ester-like odor. Based on diketone content, such solutions at pH 4 to 5 are completely stable at 5° for a week, and are 10 per cent decomposed in 3 weeks. At 28° ethyl triacetate solutions (0.005 to 0.01 M) are stable for 16 hours at pH 4.5 but are 35 to 40 per cent decomposed at pH 7.2. Triacetic acid, triacetic lactone, or acetoacetic acid did not appear to be products of the decomposition.

o-Phenylenediamine Reaction—Triacetic ester reacts directly with *o*-phenylenediamine and hence must have a β-diketone group. In the direct reaction with *o*-phenylenediamine the ester yields 80 to 82 per cent of the color obtained from equimolar amounts of acetylacetone or triacetic acid, and an initial lag in color development is noted. After distillation from 2.5 N H_2SO_4, the lag in color development is no longer noted and the final color intensity is equivalent to that of acetylacetone.

Saponification of Ethyl Triacetate—For the isolation of triacetic acid, a rather extensive preliminary study was necessary to determine the proper conditions for the hydrolysis of ethyl triacetate, since β,δ-diketones, including acetylacetone (12), are readily split by alkali. For proper interpretation of later metabolic experiments it was therefore necessary to rule out the presence of products other than triacetic acid, and to isolate the latter rather than to assume its presence after hydrolysis, as is commonly done with more stable compounds.

To evaluate the results of different methods of saponification, both the catalytic decarboxylation method and the *o*-phenylenediamine color reaction were employed. Since the ester is not decarboxylated, the appearance of β-keto acids can be determined by the decarboxylation reaction, and failure to obtain equivalent amounts of β,δ-diketones by the color reaction was assumed to be due to side reactions involving loss of the diketone group. Acetylacetone gives the color reaction but cannot be decarboxylated, while triacetic lactone does not give the direct color reaction.

TABLE I

Saponification of Triacetic Ester

"Free" diketone determined by direct *o*-phenylenediamine color reaction; "total" diketone after acid hydrolysis (8). CO_2 determined by the aniline citrate decarboxylation method. Amount of alkali present, 2 moles for each mole of triacetic ester used.

Triacetic ester ("total" diketone)	Hydrolysis temperature	Hydrolysis time	"Free" diketone after hydrolysis	Diketone loss	CO_2	Ratio	
						CO_2 to "free" diketone after hydrolysis	CO_2 to initial "total" diketone
micromoles	*°C.*	*hrs.*	*micromoles*	*per cent*	*micromoles*		
910	30	18	615	32	660	1.07	0.73
91	30	18	67	26	64	0.96	0.70
910	5	36	910	0	790	0.87	0.87
91	5	36	91	0	75	0.83	0.83
880	5	24	816	7	710	0.87	0.81
1300	5	24	1240	5	1060	0.86	0.82
			49.8*		40.2	0.81	

* 7.1 mg. or 49.2 micromoles of isolated triacetic acid.

In Table I are recorded some typical saponification results with two different concentrations of triacetic ester at 5° and 30°. The terms "total" and "free" diketones are employed, since triacetic ester yields only 80 to 82 per cent as much color as triacetic acid in the direct color reaction, while after acid hydrolysis it yields the same amount of color per mole as the free acid (8). "Free" diketone is therefore the value obtained by the direct reaction, "total" the value obtained after acid hydrolysis. During saponification at 5° the free diketone content rose until it was equal to the original "total" diketone, and simultaneously a β-keto acid was produced which with aniline citrate released CO_2 equivalent to 83 to 87 per cent of the final diketone content of the solution. Thus there was no loss of diketone, although the low CO_2 equivalent was puzzling until it was found that the isolated triacetic acid also yielded only 81 per cent of the theoretical CO_2 on catalytic decarboxylation. In contrast, at 30° there is approximately a 30 per cent loss of diketone, and the apparent equivalence of the CO_2 released and the diketone content of the solution is evidently due to formation of other β-keto acids under these conditions.

After 36 hours saponification at 5°, 80 per cent of the acid could be extracted with ethyl ether, and 50 per cent of the acid was recovered after evaporation of the solvent and drying.

Properties of Triacetic Acid

Triacetic acid is soluble in water, chloroform, ethyl ether, dioxane, and ethyl acetate, but insoluble in petroleum ether and ligroin. The acid gives a red color with ferric chloride. The acid is best stored at 5° *in vacuo* over H_2SO_4. Aqueous solutions of the acid are quite unstable. In 3 hours at 38°, 0.004 M solutions were 36 per cent decomposed over the pH range of 4 to 8, and even at 5° in 0.02 M solution there was a 10 to 15 per cent decomposition at pH 8.0. Based on diketone analysis and catalytic decarboxylation, this decomposition appears to be principally a spontaneous decarboxylation. Solutions of triacetic acid for metabolic experiments are therefore prepared just prior to use.

In Fig. 1 are recorded the ultraviolet absorption curves of triacetic acid and triacetic lactone. It may be noted that the absorption maximum of the lactone is shifted approximately 5 mμ toward the visible region, and that its absorption coefficient is slightly more than twice that of the free acid.

o-Phenylenediamine Reaction—Triacetic acid reacts directly with *o*-phenylenediamine to produce the typical red color. An initial lag in color development is noted as compared with acetylacetone, and under comparable conditions in the respirometer it is clear that the acid undergoes decarboxylation during the period of color development. The same color value is reached as with acetylacetone.

Decarboxylation of Triacetic Acid—Studies were made of the catalytic decarboxylation of triacetic acid compared with acetoacetic acid. Triacetic acid was decarboxylated by aniline citrate (5), aniline acetate (7), aniline acetate in the presence of sodium cyanide (6), *o*- and p-phenylenediamine citrate (7), and by low concentrations of *o*-phenylenediamine (250 mg. per cent) in dilute H_2SO_4 (pH 1.3 to 1.5). The decomposition of triacetic acid by aniline citrate is considerably slower than that of acetoacetic acid, but the reverse is true with *o*-phenylenediamine in dilute sulfuric acid. With the other amine catalysts the rates were roughly equivalent.

After completion of the triacetic acid decarboxylation by aniline citrate, only 81 to 85 per cent of the theoretical CO_2 had been released, and 86 to 89 per cent by *o*-phenylenediamine citrate compared to a 90 to 95 per cent yield of CO_2 from acetoacetic acid. After completion of the decomposition of triacetic acid with aniline citrate, 90 to 95 per cent of "free" diketone was present, indicating that formation of triacetic lactone was not a significant side reaction during the decarboxylation.

Copper sulfate or aluminum chloride, which catalytically remove one carboxyl from dicarboxylic β-keto acids such as oxalacetic acid or acetonedicarboxylic acid (13), does not decarboxylate triacetic acid. On the other hand, triacetic acid is decarboxylated by aniline acetate in the presence of sodium cyanide; under these conditions oxalacetic acid is not attacked (6).

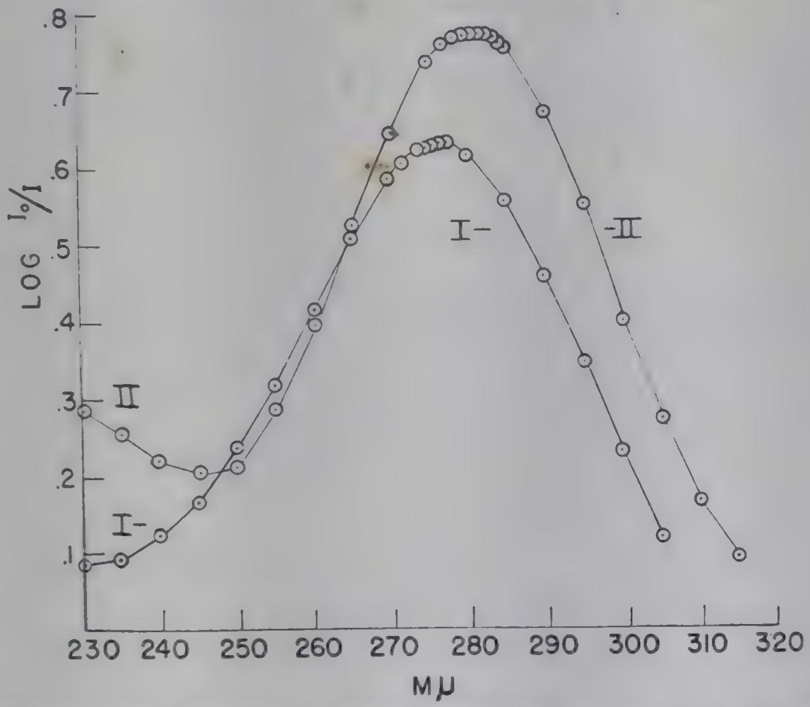


FIG. 1. Ultraviolet absorption spectra of triacetic acid (2×10^{-4} M, Curve I) and triacetic lactone (1×10^{-4} M, Curve II) in water. Beckman spectrophotometer; 1 cm. cells.

Triacetic Lactone—In contrast to the free acid, triacetic lactone is remarkably stable. A 0.04 M solution of the lactone was not affected by 1 M alkali at 30° for 16 hours, nor by 0.2 M alkali at 60° for 1 hour. Solutions so treated gave no direct color reaction with *o*-phenylenediamine and were not decarboxylated with aniline citrate, but gave quantitative diketone (acetylacetone) recovery after hydrolysis with sulfuric acid. In this connection the analyses of all the salts of triacetic lactone prepared by Collie (9) correspond to those of the enol lactone and not to those of the free acid. Our studies also indicate that the lactone and free acid are not readily interconvertible nor in an equilibrium state in solution.

SUMMARY

Triacetic acid (β,δ-diketohexanoic acid) was synthesized through a series of intermediates of which triacetic lactone (9) and copper ethyl triacetate (4) have been previously described. Triacetic acid is a light yellow, waxy solid melting at 29–31°, soluble in water and several organic solvents, and unstable in aqueous solution. Since triacetic acid is a β-keto acid, it is decarboxylated by aromatic amines, and because of its β-diketone group reacts with *o*-phenylenediamine to produce a red color which is used for quantitative estimation of the acid and its derivatives. Other properties of triacetic acid, triacetic ester, and the lactone are recorded.

BIBLIOGRAPHY

1. Stotz, E., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **5**, 129 (1945).
2. Breusch, F. L., *Enzymologia*, **11**, 169 (1944).
3. Breusch, F. L., and Ulusoy, E., *Arch. Biochem.*, **14**, 183 (1947).
4. Sproxton, F., *J. Chem. Soc.*, **89**, 1186 (1906).
5. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **39**, 408 (1945).
6. Dewan, J. G., and Green, D. E., *Biochem. J.*, **31**, 1074 (1937).
7. Edson, N. L., *Biochem. J.*, **29**, 2082 (1935).
8. Witter, R. F., Snyder, J., and Stotz, E., *J. Biol. Chem.*, **176**, 493 (1948).
9. Collie, J. N., *J. Chem. Soc.*, **59**, 607 (1891).
10. Clift, F. P., and Cook, R. P., *Biochem. J.*, **26**, 1800 (1926).
11. Knorr, L., *Ber. chem. Ges.*, **26**, 100 (1893).
12. Combes, A., *Ann. chim. et phys.*, **12**, 229 (1887).
13. Krebs, H. A., *Biochem. J.*, **36**, 303 (1942).

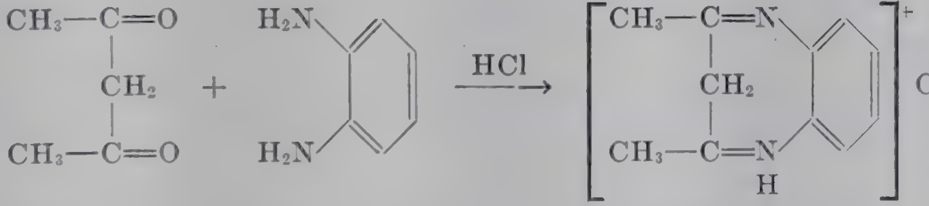
COLORIMETRIC DETERMINATION OF ACETYLACETONE AND RELATED β-DIKETONES*

By ROBERT F. WITTER,† JEANETTE SNYDER, AND ELMER STOTZ†

(From the Biochemical Laboratories, Division of Food Science and Technology, New York State Agricultural Experiment Station, Cornell University, Geneva, and the Department of Biochemistry, School of Medicine and Dentistry, The University of Rochester, Rochester, New York)

(Received for publication, July 8, 1948)

There is a lack both of the intermediates often proposed in fatty acid oxidation and synthesis and of methods for their analysis. In the preceding paper (1) the synthesis of triacetic acid (β, δ-diketohexanoic acid) was reported; in this paper is described the colorimetric determination of this compound and related β-diketones. The method is sensitive, quite specific, and is free from interference by acetoacetic acid and acetone. The color reaction involves a condensation of the diketone with *o*-phenylenediamine in acid solution to produce a reddish purple color, a reaction previously described (2–4). In the case of acetylacetone, which has served as a convenient standard, the reaction may be written as in the accompanying scheme.



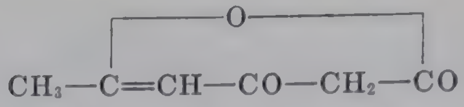
2,4-Dimethyl(1,5)benzodiazapine hydrochloride

In the reaction with triacetic acid, the acid appears to be decarboxylated to acetylacetone during the period of color development (1). In the case of ethyl triacetate the ester itself probably reacts with the *o*-phenylenediamine or at least is only partially hydrolyzed during color development, since the color yield per mole of ester is less than with acetylacetone or the free acid. Triacetic lactone

---

* Journal Paper No. 751, New York State Agricultural Experiment Station, Cornell University, Geneva, New York. This research was supported by grants from the Nutrition Foundation, Inc., the Sugar Research Foundation, Inc., and the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service.

† Present address, School of Medicine and Dentistry, The University of Rochester.



$$\mathrm{CH_3{-}C{=}CH{-}CO{-}CH_2{-}CO}$$

which lacks the β-diketone linkage does not react directly with the reagent at room temperature, but can be converted to acetylacetone by hot acid hydrolysis. A number of qualitative tests for the identification of the purple pigment 2,4-dimethyl(1,5)benzodiazapine are reported, and the extent of interference of various substances determined. The recovery of acetylacetone or triacetic lactone added to various biological systems has been tested. From 2 to 8 micromoles of diketone are recovered to the extent of 95 to 100 per cent.

EXPERIMENTAL

Chemicals and Reagents—Sodium pyruvate was prepared by the method of Robertson (5), and oxalacetic acid by the procedure given by Cohen (6). Triacetic lactone was prepared from dehydroacetic acid by the method of Collie (7). The identification and analysis of this compound are given in the preceding paper (1). The usual grades of ethyl acetoacetate had to be redistilled twice *in vacuo* to remove an impurity that forms a reddish purple color with *o*-phenylenediamine.

1. *Stock acetylacetone.* 1.00 gm. of acetylacetone (freshly distilled *in vacuo*) is dissolved in 500 ml. of 0.1 M potassium phosphate buffer of pH 7.4, and diluted to 1000 ml. This solution keeps for at least 1 month at 5° in the dark.

2. *Phosphate-sulfuric acid reagent.* Dissolve 43.5 gm. of K_2HPO_4 and 20 ml. of 5 N phosphoric acid in 500 ml. of 2.4 N sulfuric acid and dilute to 1000 ml.

3. *o-Phenylenediamine reagent.* Dissolve 100 mg. of *o*-phenylenediamine in 25 ml. of phosphate-sulfuric acid reagent. This reagent should be prepared just prior to use.

Method A (*Direct or "Free" Diketone*)—This method is used to estimate acetylacetone, triacetic acid, or ethyl triacetate.

To 12 ml. of test solution containing from 2 to 8 micromoles of diketone add 2 ml. of *o*-phenylenediamine reagent, mix, and allow to stand at room temperature for 30 minutes. Compare the color against a reagent blank containing 12 ml. of water and 2 ml. of *o*-phenylenediamine reagent at 500 mμ in a photoelectric colorimeter.

If the test solution is colored or turbid, a blank should be run containing the same amount of test solution and 2 ml. of phosphate-sulfuric acid reagent, and its color value (read against water) subtracted from that of the test solution with *o*-phenylenediamine.

Method B (*"Total" Diketone, Triacetic Lactone*)—This method is used principally for the analysis of triacetic lactone but is also applicable to ethyl triacetate. It involves a conversion of either compound to acetylacetone; hence in the determination of either compound a standard acetylacetone curve may be used. In the *direct* method for triacetic ester a factor must be employed. Method B may be used to determine "total" diketone when triacetic lactone is present with "free" diketones. The difference obtained in Methods A and B can be used as a measure of triacetic lactone.

To the distillation flask of a unit such as that described by Stotz (8) are added 13 ml. of test solution containing from 2 to 8 micromoles of diketone, 1 ml. of concentrated sulfuric acid, and a quartz pebble (a few crystals of cholesterol are most useful to prevent foaming during the boiling of filtrates of biological material). The flask is placed in a small heated sand bath to provide a slow, steady boiling so that 10 to 11 ml. of distillate are collected during the course of 15 to 20 minutes. A 25 ml. glass-stoppered cylinder immersed in an ice bath serves as a convenient receiving vessel. At the end of the distillation, the contents of the graduate are diluted to 12 ml. 2.0 ml. of *o*-phenylenediamine reagent are added, the solution mixed, and the color allowed to develop for 30 minutes as in the *direct* method.

Qualitative Tests for Dimethylbenzodiazapine—The following qualitative tests for the typical purple pigment may be found convenient to identify the reaction. In 10 ml. of the colored reaction mixture obtained in the quantitative determination, (1) the color is discharged immediately by the addition of 2 ml. of 10 per cent NaOH, and in 20 to 30 minutes by 40 per cent formaldehyde, 2 M hydroxylamine hydrochloride, or 0.5 M sodium bisulfite, (2) the addition of acid to approximately pH 2.0 restores the color of the solution which has been made alkaline, (3) the leuco pigment is extracted from alkaline solution with chloroform, and the purple color reappears when glacial acetic acid is added, and (4) the purple pigment is quantitatively precipitated with 0.3 ml. of 10 per cent sodium tungstate at pH 1.3 to 2.0. (An orange precipitate separates with the crotonaldehyde-*o*-phenylenediamine compound, and a white precipitate with the diacetyl-*o*-phenylenediamine compound.)

Diketone Color Values—In the *direct* analytical method for diketones a straight line relation was found between color density and concentration of the diketone. This is recorded in Table I, along with the relative color values obtained with the other diketones in the direct reaction. It is evident that triacetic acid yields the same color per mole as acetylacetone, but ethyl triacetate has a lower color yield. Nevertheless a straight line relationship of density to concentration is still obtained with the ester.

Effect of pH and o-Phenylenediamine Concentration—The effects of changes in the pH and *o*-phenylenediamine concentration on the color reaction with acetylacetone are illustrated in Table II. Acetylacetone (8 micromoles) samples were treated with 2 ml. of *o*-phenylenediamine of various acid strengths and concentrations of diamine. The color density

TABLE I

Relative Color Value Obtained in o-Phenylenediamine Reaction by Different Diketones

Color reaction made according to Method A. Two different preparations of triacetic acid gave identical values. The amount of triacetic ester added was measured from the "total" diketone content according to Method B.

Amount added	Color value found						
	Acetylacetone		Triacetic acid		Ethyl triacetate		Triacetic lactone
micromoles	*micromoles**	*ratio†*	*micromoles*	*ratio*	*micromoles*	*ratio*	
2.0	2.01	1.01	2.00	1.00	1.66	0.83	0
4.0	4.00	1.00	4.04	1.01	3.22	0.81	0
6.0	6.00	1.00	6.10	1.02	4.90	0.82	0
8.0	7.95	0.99	8.00	1.00	6.47	0.81	0

* As micromoles of acetylacetone determined from the standard curve.

† Calculated as the micromoles found divided by the micromoles of substances added.

TABLE II

Effect of o-Phenylenediamine Concentration and pH on Intensity of Color Reaction

8 micromoles of acetylacetone used in each sample.

pH	*o*-Phenylenediamine	Minimum time of color development	$K \times 10^{-2}$*
	mg.	*min.*	
1.3	2	60	7.01
1.3	4	20	7.90
1.3	8	20	8.35
0.3	4	10	6.50
1.2	4	20	7.80
2.1	4	60	7.70
3.1	4	90	6.90
0.3	8	10	7.60
1.2	8	20	8.30
2.1	8	30	8.30
3.0	8	60	7.70

* $K = (2 - \log G)/C$, where G = the galvanometer reading at 500 mμ when the control tube is set at 100, and C = the molar concentration of acetylacetone.

was estimated at 10 minute intervals against the appropriate *o*-phenylenediamine blank.

With either 4 or 8 mg. of *o*-phenylenediamine the color density was essentially constant between 1.3 and 2.0 but was decreased when the pH of the solution was decreased to 0.3 or increased to 3.0. Thus the pH and diamine concentration must be controlled, although straight line relations were obtained between color density and diketone concentration except with the smallest amount of *o*-phenylenediamine. The conditions chosen and described under Method A permit the greatest variation in pH and time of color development without affecting the color value.

Hydrolysis and Recovery of Triacetic Lactone—The strength of acid and time for distillation recommended in Method B are critical for successful recovery of triacetic lactone. Only 15 to 20 per cent of the lactone was hydrolyzed when 1 ml. of 10 per cent metaphosphoric acid was used in place of 1 ml. of sulfuric acid, and 74 to 78 per cent was hydrolyzed when 0.5 ml. of sulfuric acid was employed. About 74 to 88 per cent recovery was obtained if the time of distillation was lowered to 8 to 10 minutes; hence the fairly slow steady distillation recommended is essential. Simultaneous separation of the acetylacetone formed upon hydrolysis of the lactone seemed essential, since separation of the hydrolysis and distillation steps did not give satisfactory yields.

TABLE III

Recovery of Triacetic Lactone from Pure Solution

Compound added		Compound recovered			
Lactone	Acetylacetone	Lactone*		Acetylacetone†	
micromoles	*micromoles*	*micromoles*	*per cent*	*micromoles*	*per cent*
2.00		1.97	98		
4.00		3.90	98		
8.00		7.70	96		
4.00	2.00	3.74‡	94	1.98	99
2.00	5.00	1.84‡	92	4.95	99
2.00	2.00	1.80‡	90	2.00	100

* Determined by acid distillation (Method B).

† Determined by Method A.

‡ Calculated from the difference in color density of equal aliquots determined by Methods A and B.

With the conditions recommended, 96 to 98 per cent recoveries of triacetic lactone have been obtained. Typical results are recorded in Table III. In practice, duplicate samples have checked within 2 per cent. At least some of the lowered yield in the determination of triacetic lactone is probably due to destruction of the acetylacetone liberated in the strong acid solution, since a 2 to 5 per cent loss was observed if acetylacetone was substituted for the lactone. Table III also demonstrates that triacetic lactone can be estimated in the presence of acetylacetone, and here the apparently lowered yield of triacetic lactone is evident, owing for the most part to acid destruction of the free acetylacetone present.

The distillation procedure may be convenient for analysis of acetylacetone or triacetic acid if non-volatile interfering substances are known to be present.

Specificity and Interfering Compounds—No color was formed with *o*-phenylenediamine under the conditions of Method A with 100 micromoles of the following compounds: diacetyl, acetoin, acetone, acetaldehyde, succinic acid, fumaric acid, acetoacetic acid, oxalacetic acid, levulinic acid, citric acid, ethyl acetoacetate, ethyl levulinate, or diethyl acetonedicarboxylate. Since these compounds were tested in a concentration over 10 times that of the acetylacetone, the color reaction appears to be specific. On the other hand the color reaction is inhibited by certain substances. Tungstic acid precipitates the pigment at pH 1.3 to 2.0, oxidizing agents such as dichromate or peroxide oxidize the *o*-phenylenediamine, and bisulfite, hydroxylamine, or semicarbazide inhibits color formation. An inhibition of the color reaction of 10 to 15 per cent was observed in the presence of 100 micromoles of pyruvic acid (used as the purified sodium salt) and a complete inhibition occurred with 100 micromoles of diacetyl or formaldehyde. Reduction of pyruvate to 50 micromoles and diacetyl to 10 micromoles results in a 93 to 95 per cent color yield with acetylacetone. 100 micromoles of C.P. ascorbic acid did not cause an inhibition of color development, but some samples of U. S. P. ascorbic acid at a level of 50 micromoles caused a 10 to 15 per cent inhibition. Glucose in 10 per cent solution or 0.4 M urea did not interfere in the direct determination of acetylacetone.

Crotonaldehyde in amounts of from 10 to 50 micromoles gives an instantaneous yellow to orange color upon addition of *o*-phenylenediamine. Thus when 50 micromoles of crotonaldehyde are present, 2 micromoles of acetylacetone appear to give a 135 per cent yield, 4 micromoles a 115 per cent yield, and 8 micromoles a 105 per cent yield. The crotonaldehyde interference can be eliminated by the addition of 2 ml. of 0.05 M sodium bisulfite to 10 ml. of the solution after the usual 20 to 30 minutes of color development when the color is read after 15 to 20 minutes against a reagent blank containing bisulfite. Bisulfite causes a decrease of 40 per cent in the color owing to acetylacetone but a straight line relationship between color density and acetylacetone concentration is still observed.

With certain exceptions the effects of the interfering substances are the same in the triacetic lactone distillation procedure as in the direct acetylacetone method. High concentrations of glucose apparently yield volatile split-products during the hot acid distillation which give orange to red colors with *o*-phenylenediamine. Interference is not serious, however, until 1 per cent glucose is present. Low recoveries (67 to 70 per cent) of triacetic lactone were observed in the presence of 0.4 M urea, a concentration which may be found in urine. The same effect was observed in the distillation of acetylacetone from sulfuric acid solution but not from metaphosphoric acid. Since urea or its distillation products do not interfere with the direct color reaction, it appears that urea condenses with acetylacetone under the hot acid conditions necessary for the hydrolysis of triacetic lactone.

Although Elson and Morgan (9) reported a method for the estimation of glucosamine based on its condensation with acetylacetone in hot acid solution to form pyrrole derivatives, 50 micromoles of glucosamine did not interfere in the determination of triacetic lactone.

Table IV

Recovery of Acetylacetone and Triacetic Lactone Added to Biological Materials

Biological material	Triacetic lactone			Acetylacetone		
	Added	Recovered		Added	Recovered	
	micromoles	*micromoles*	*per cent*	*micromoles*	*micromoles*	*per cent*
10% kidney mince	3.20	3.14	98			
	6.40	6.10	95			
				4.24	4.10	97
				2.14	2.02	94
20% glucose "residual harvest medium"				2.22	2.15	97
				4.22	4.06	96
				8.10	7.90	98
8% *Fusarium lycopersici* suspension	2.50	2.40	96			
				2.50	2.36	94
Extract of *Fusarium lycopersici*	2.00	1.90	95			
	6.40	6.25	98			
Suspension of *Escherichia coli*				4.00	4.00	100
0.2% peptone "residual harvest medium"	4.00	3.75	94			
Urine, direct procedure				5.26	5.35	102
				9.26	9.26	100
" distillation procedure without sulfuric acid				8.00	7.85	98

Recovery of Diketones from Biological Systems—In Table IV are presented results in which different amounts of acetylacetone or triacetic lactone were added to various biological systems. Metaphosphoric acid filtrates were preferred for general use, with 2 per cent acid as a final concentration. In the acetylacetone determination, if the metaphosphoric acid filtrate was obviously turbid or colored, a blank in addition to the *o*-phenylenediamine control was run. In this control, phosphate-sulfuric acid reagent was substituted for *o*-phenylenediamine as recommended under Method A. An internal blank may be found convenient in some cases by discharging the color with 2 ml. of 40 per cent formaldehyde and estimating the residual color density.

In none of the biological materials analyzed was there any evidence for a measurable amount of acetylacetone, triacetic acid, or triacetic lactone occurring naturally. The systems used were representative of the types in which triacetic acid metabolism might be studied, and provided for the testing of possible interfering materials. The biological systems tested included (*a*) 10 per cent kidney mince, (*b*) a 10 per cent glucose medium (10) from which a high fat mycelial mat of *Fusarium lycopersici* (11) had been harvested (called "residual harvest medium" in Table IV), (*c*) an 8 per cent cell suspension of *F. lycopersici*, (*d*) an extract of *F. lycopersici* prepared by grinding 5 gm. of lyophilized mold with powdered glass (12) and suspending in 100 ml. of water, (*e*) a suspension of *Escherichia coli* (20 mg. of dry weight per ml.), (*f*) a peptone medium (13) from which *Escherichia coli* was collected after 48 hours of aerobic growth at 37°, and (*g*) urine. Satisfactory recoveries of both acetylacetone and triacetic lactone were found with these biological materials.

SUMMARY

Methods are presented for the colorimetric determination of from 2 to 8 micromoles of the β-diketones: acetylacetone, triacetic acid, ethyl triacetate, and triacetic lactone. The lactone is first converted to acetylacetone by acid hydrolysis and separated by distillation. The color reaction depends on a condensation of the diketones in acid solution with *o*-phenylenediamine to form the reddish purple dimethylbenzodiazapine. The specificity of the reaction has been studied and the limiting concentrations of interfering substances determined. Recoveries of acetylacetone and triacetic lactone added to a variety of biological systems are given.

BIBLIOGRAPHY

1. Witter, R. F., and Stotz, E., *J. Biol. Chem.*, **176**, 485 (1948).
2. Thiele, J., and Steimmig, G., *Ber. chem. Ges.*, **40**, 955 (1907).
3. Vaisman, S. B., *Tr. Inst. Chem. Kharkov Univ.*, **4**, 157 (1938); *Chem. Abstr.*, **34**, 5847 (1940).
4. Vaisman, S. B., *Trudy Inst. Khim. Kharkov, Gosudarst. Univ.*, **5**, 57 (1940); *Chem. Abstr.*, **38**, 750 (1944).
5. Robertson, W. B., *Science*, **96**, 93 (1942).
6. Cohen, P. P., *J. Biol. Chem.*, **136**, 565 (1940).
7. Collie, N. F., *J. Chem. Soc.*, **59**, 607 (1891).
8. Stotz, E., *J. Biol. Chem.*, **148**, 585 (1943).
9. Elson, L. A., and Morgan, W. T. J., *Biochem. J.*, **27**, 1824 (1934).
10. Lockwood, L. B., Ward, G. E., May, O. E., Herrick, H. T., and O'Neil, T., *Zentr. Bakt., 2. Abt., Ref.*, **90**, 411 (1934).
11. Witter, R. F., and Stotz, E., *Arch. Biochem.*, **9**, 331 (1946).
12. Wiggert, W. P., Silverman, M., Utter, M. F., and Werkman, C. H., *Iowa State Coll. J. Sc.*, **14**, 179 (1940).
13. Lehninger, A. L., *J. Biol. Chem.*, **143**, 147 (1942).

THE METABOLISM IN VITRO OF TRIACETIC ACID AND RELATED DIKETONES*

By ROBERT F. WITTER and ELMER STOTZ
With the Technical Assistance of Vonnie Burkitt
(*From the Department of Biochemistry, School of Medicine and Dentistry, The University of Rochester, Rochester, New York*)

(Received for publication, July 8, 1948)

Polyketonic acids have been postulated as intermediates in theories of fatty acid oxidation (1–3) and synthesis (4), but direct evidence has been generally lacking. The α,γ-diketo acid, acetopyruvic acid, is apparently rapidly metabolized by animal tissues (5, 6), and an enzyme which produces pyruvic acid from this compound has been partially purified by Meister and Greenstein (7). However, Lehninger (8) found that α,γ-diketooctanoic acid was only slowly metabolized by broken cell preparations of liver, in contrast to octanoic acid itself, and the study of Weinhouse and coworkers (9) with carboxyl-labeled octanoic acid leave little place for α,γ oxidation as a mechanism for the oxidation of fatty acids with an even number of carbon atoms.

β oxidation is of course the more generally accepted basis for fatty acid oxidation. It may be pointed out, however, that none of the existing theories of fatty acid oxidation is based on evidence which precludes multiple oxidation of the fatty acid before splitting, but that the conflicting theories differ mainly in the suggested mode of cleavage of the oxidized fatty acids or in subsequent reactions. Polyketonic acids, resulting from successive condensations of acetic acid molecules, might also be postulated as intermediates in fat synthesis.

In spite of the probable importance of multiple alternate β-oxidized fatty acids in fat metabolism, representatives of this class of compounds have not been available for testing. In the preceding papers (10, 11) the preparation and determination of triacetic acid (β,δ-diketohexanoic acid) have been described. In this report it will be shown that triacetic acid is readily metabolized by liver tissue *in vitro*, with the formation of acetoacetic acid and acetic acid, a finding which necessitates the consideration of such "multiple alternate" oxidized fatty acids in theories of fat metabolism.

During the course of our investigations, Breusch and Ulusoy (12) reported that the δ-lactone of triacetic acid (referred to by these investigators

---

* This research was supported by grants from the Nutrition Foundation, Inc., the Sugar Research Foundation, Inc., and the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service.

as "triacetic acid") was metabolized by liver at such a rate as to make it possible that this substance is an intermediate in fatty acid oxidation. These investigators found that approximately 1.3 moles of acetoacetic acid were formed from 1 mole of the lactone, a finding which might support β splitting and recondensation of 2-carbon fragments as a mechanism of hexanoic acid oxidation. Free acetic acid could not be found as a product of the reaction.

Analytical Methods—Since the β-diketones were found to interfere in the several methods tested for the determination of acetone, it was first necessary to develop a procedure for the preliminary removal of these compounds. This was accomplished by precipitation of the colored acetylacetone-*o*-phenylenediamine complex with tungstic acid. Acetone was then determined by a modification of the vanillin method (13) which seemed to offer the greatest specificity.

Removal of Acetylacetone Prior to Acetone Analysis—Metaphosphoric acid filtrates containing both acetoacetic acid and triacetic acid are distilled, and the *o*-phenylenediamine color reaction for acetylacetone is carried out on the filtrate as described previously (11). Acetone present in the filtrate does not react with the *o*-phenylenediamine reagent. For removal of the colored complex, 0.3 ml. of 10 per cent sodium tungstate is then added, and the solution allowed to stand overnight at 5°. After the centrifuged precipitate is discarded, 10 ml. of the colorless solution are distilled into 1 ml. of water contained in a 25 ml. glass-stoppered cylinder placed in an ice bath. Approximately 8 ml. of distillate are collected, and the volume adjusted to 10 ml. A 1 to 2 ml. portion of this filtrate is used for the vanillin reaction to determine acetone, as described below.

In practice both diketones and acetone can be determined on the same tissue filtrate by measuring the intensity of the *o*-phenylenediamine color reaction in the distillate, followed by removal of the colored complex with tungstate and estimation of acetone.

Vanillin Reaction—The following reagents were employed: (1) potassium hydroxide, 100 gm. dissolved in 60 ml. of distilled water; (2) 10 per cent vanillin (Eastman, practical grade) in acetone-free methyl alcohol, prepared fresh for use; (3) acetone stock standard, 0.01 M acetone, standardized by the iodoform reaction; this solution keeps at least for a month at 5°; (4) acetone working standard, 0.1 to 0.5 micromole of acetone per ml., freshly prepared.

Procedure—To 2 ml. of acetone solution (0.2 to 1.0 micromole of acetone) contained in a test-tube are added 2 ml. of potassium hydroxide, with cooling in an ice bath. After the tube is removed from the ice bath, 1 ml. of vanillin reagent is added and the tube shaken for thorough mixing. The gelatinous precipitate formed dissolves on subsequent heating. A clean rubber stopper is inserted, and the tube heated at 65° for 20 minutes. After cooling, 10 ml. of water are added, the tube contents mixed, and the color allowed to develop for 15 minutes. The color may be estimated by a Lumetron colorimeter with the 515 mμ filter or by the Klett-Summerson colorimeter with the green filter. A vanillin "control" without acetone is employed as well as "standard" tubes containing from 0.2 to 1.0 micromole of acetone.

Under a given set of conditions a linear relation between acetone concentration and color density is obtained, but standard curves may vary as much as 10 per cent; hence standards are always employed with a set of unknowns. Methyl ketones other than acetone do not give the vanillin reaction, but acetylacetone gives the same color per mole. Acetylacetone is apparently converted to acetone by alkali, a reaction previously noted (14). After removal of the colored acetylacetone-*o*-phenylenediamine complex with tungstic acid, as previously described, the distillates from such treatments still yield a slight blank in the vanillin reaction, equivalent to 0.0 to 0.03 micromole of acetone. This blank was regularly deducted in experiments involving both diketones and acetone, but always represented less than 5 per cent of the acetone present.

In Table I are presented the results of acetone recovery, alone and in the presence of acetylacetone.

Triacetic acid, ester, and lactone were determined by the methods previously described (11), total β-keto acids by the aniline citrate decarboxylation method (15), and β-hydroxybutyric acid by a modification of the Barnes and Wick method (16).

Utilization of Triacetic Acid, Ester, and Lactone by Tissue Homogenates

Rats were fasted overnight, sacrificed, and tissue homogenates prepared by grinding 1 part of tissue with 3 parts of isotonic saline at 5° for 5 minutes in a mechanical glass homogenizer (17). The usual reaction mixture was composed of 0.5 ml. of 0.01 M substrate, 0.2 ml. of 0.1 M sodium phosphate buffer (pH 7.4), 0.4 to 0.8 ml. of tissue homogenate, and distilled water to make a volume of 1.5 ml. Such mixtures were incubated in stoppered tubes at 30° with shaking. At zero time and at various intervals thereafter, 6 ml. of 5 per cent metaphosphoric acid were added to stop the reaction, and the mixture diluted to 15 ml. Analyses were carried out on the supernatant fluids. Since a linear relationship was found between substrate disappearance and time of the reaction, it was customary to determine the substrate concentration at zero time and at two subsequent time intervals. The results are reported as micromoles of triacetic compound which disappeared per ml. of tissue preparation per hour. Several representative experiments are reported in Table II.

TABLE I

Recovery of Acetone Alone and in Presence of Acetylacetone

Acetylacetone added	Acetone	
	Added	Found
micromole	*micromole*	*micromole*
0	0.56	0.56
0	0.42	0.42
0	0.28	0.30
0.14	0.00	0.01
0.14	0.56	0.54
0.14	0.42	0.42
0.14	0.28	0.30
0.35	0.00	0.02
0.35	0.56	0.57
0.35	0.42	0.44
0.35	0.28	0.26

TABLE II

Metabolism of Triacetic Acid, Ester, and Lactone by Tissue Homogenates

Tissue	Time of incubation	Triacetic substrate	Activity*	Ratio†
	min.			
Rat liver	40	Acid	12.1	0.90
“ “	40	Ester	10.8	0.95
“ “	80	Lactone	1.3	0.90
“ “	45	Acid	11.6	1.00
“ “	45	Ester	9.8	1.05
“ “	90	Lactone	0.9	0.90
“ “	45	Acetylacetone	0	
“ “	45	Acid		1.00
“ “	45	Ester		0.90
“ “	90	Lactone	1.3	0.95
“ “	30	Acid	9.0	1.00
“ “	45	Acetoacetate	0	
“ kidney	30	Acid	5.9	1.05
Beef liver	60	“	3.6	
“ “	60	Lactone	0	
Rabbit liver	40	Acid	5.9	
“ “	120	Lactone	0.8	
“ kidney	40	Acid	1.1	
“ “	120	Lactone	0.4	

* Micromoles of substrate which disappeared per ml. of homogenate employed per hour.

† Micromoles of acetoacetate formed per micromole of triacetic substrate which disappeared.

It may be seen that triacetic acid and ester were metabolized by rat liver homogenate at approximately the same rate, while triacetic lactone disappeared at only about one-eighth this rate. The rate of disappearance of the substrates was not affected by the absence of oxygen, nor by the addition of adenosine triphosphate, Mg^{++}, or diphosphopyridine nucleotide. In connection with these findings, the relative rates of disappearance of triacetic acid and hexanoic acid have been compared. Homogenates with appropriate additions according to Lehninger (18) caused a triacetic acid disappearance about 1.5 times as rapid as that of hexanoic acid.

Triacetic acid was not metabolized by rat heart, skeletal muscle, spleen, or pancreas.

Formation of Acetoacetic and Acetic Acids from Triacetic Acid in Rat Liver Homogenates—A determination of the acetoacetic acid formed during the metabolism of triacetic compounds in liver was also made on the same reaction mixture. These results are also recorded in Table II.

1 mole of acetoacetic acid was regularly formed from 1 mole of triacetic acid, ester, or lactone. This was true whether 50 per cent or 90 per cent of the triacetic acid had disappeared. Added acetoacetic acid or acetylacetone did not disappear from the reaction mixture, nor was any β-hydroxybutyric acid formed from triacetic acid. The same ratio of acetoacetic acid formed to the triacetic compound which disappeared was observed in the presence of adenosine triphosphate, Mg^{++}, and cytochrome *c*, conditions which provided for an active oxidation of hexanoic acid with acetoacetate formation.

There seemed to be no alternative than acetic acid for the other product of triacetic acid breakdown, according to the equation, $CH_3COCH_2COCH_2COOH + H_2O \rightarrow CH_3COCH_2COOH + CH_3COOH$.

The formation of acetic acid was observed in experiments conducted on a scale larger than usual, as follows: 4.0 ml. of 0.1 м phosphate buffer (pH 7.4), 16 ml. of 25 per cent rat liver homogenate, and 10 ml. of 0.02 м triacetic acid were incubated at 30° for 90 minutes. Simultaneous controls measured the formation of volatile acid in the absence of triacetic acid, and the recovery of added acetic acid. Metaphosphoric acid filtrates were prepared. The volatile fatty acids were distilled after addition of 25 gm. of magnesium sulfate (19) to 100 ml. of metaphosphoric acid filtrate, and the distillation continued until crystallization of the residue in the distilling flask was observed. The distillate was boiled for 3 to 5 minutes with almost complete removal of acetylacetone formed from the residual triacetic acid. The solution was then titrated with 0.01 м alkali. Neither acetoacetic acid nor acetylacetone interfered with the determination as described at the concentrations which occurred in these experiments. Chloride and sulfate tests on the distillates were negative.

In a typical experiment 184 micromoles of triacetic acid disappeared in 90 minutes; 164 micromoles of acetoacetic acid and 169 micromoles of volatile fatty acid were recovered. The yield of volatile fatty acid was corrected for an 85 per cent recovery of acetic acid from a liver homogenate and for "endogenous" fatty acids equivalent to about 8 per cent of the acid found in the experimental sample. Similar recoveries of acetic acid have been reported by other workers under similar analytical conditions (20, 21).

For the identification of the volatile fatty acid as acetic acid, the solution after alkali titration was evaporated to a small volume, acidified, and redistilled. The Duclaux number of the volatile fatty acid was determined parallel with samples prepared in the same way after addition of equivalent quantities of acetic acid to liver homogenates. The data in Table III show that the Duclaux number of the unknown acid is very similar if not identical with that of acetic acid, and readily distinguished, for example, from that of propionic acid. A positive lanthanum nitrate test (22) for acetic acid was also obtained on the volatile fatty acid fraction.

TABLE III

Duclaux Constants of Acid Produced from Triacetic Acid in Liver Homogenates

Acid	Per cent distilled			Total acid distilled
	Fraction 1	Fraction 2	Fraction 3	
				per cent
Unknown	22.4	25.3	34.9	82
Acetic acid added to homogenate	21.6	24.6	33.2	79
Acetic acid	20.8	24.6	33.2	79
Propionic acid	37.8	33.2	26.5	97.5

Triacetic Acid Splitting with Purified Liver Enzyme—By means of fractional alcohol precipitation and selective heat denaturation, a 100-fold purified enzyme was prepared from beef liver which catalyzed the conversion of 1 mole of triacetic acid to 2 moles of acid.[1] Acid production was followed manometrically in the Warburg apparatus with bicarbonate buffer and a gas atmosphere of 95 per cent N_2-5 per cent CO_2 at 30°, pH 7.4. The results are recorded in Table IV.

For each mole of triacetic acid which disappeared, 1 mole of acetoacetic acid and an extra mole of another acid were formed. The acetoacetic acid was determined both by the colorimetric method and the manometric decarboxylation procedure. Acetic acid was indicated here as the other acid formed by a positive lanthanum nitrate test on the volatile fatty acid fraction from the reaction mixture.

---

[1] The purification and properties of this enzyme will be the subject of a later communication.

The purified enzyme did not attack acetylacetone, triacetic ester, triacetic lactone, β-ketohexanoic acid, or acetoacetic acid.

Ethyl Triacetate Metabolism—In tracing the mechanism involved in the disappearance of triacetic ester from rat liver homogenates, the analytical problem was to determine the triacetic ester, free triacetic acid, and acetoacetic acid in the same reaction mixture. The total β-diketones (ester and acid) were estimated by the *o*-phenylenediamine procedure, and acetoacetic acid by the vanillin reaction after β-diketone removal. Total β-keto acids (acetoacetic plus triacetic) were determined by the manometric decarboxylation procedure, and from these values the triacetic ester could be calculated.

TABLE IV

Splitting of Triacetic Acid by Purified Liver Enzyme

10 micromoles of triacetic acid in 3.3 ml. volume in Warburg flasks. Bicarbonate buffer, pH 7.4; temperature 30°.

Enzyme	Time of incubation	Triacetic acid disappeared (chemical analysis)	Acetoacetic acid formed	Extra acid formed (manometric determination)
ml.	*min.*	*micromoles*	*micromoles*	*micromoles*
0.2	40	3.6	4.0	3.4
0.4	40	8.0	8.0	7.0
0.6	40	9.2	9.3	8.9
0.5	30	9.2	9.4	8.4
0.5	30	8.1	7.9	7.2

Experiments were conducted with liver homogenates, purified liver esterase,[2] and purified triacetic acid enzyme. The results recorded in Table V make it clear that the utilization of triacetic ester involves a preliminary hydrolysis of the ester by liver esterase, with subsequent splitting of the liberated triacetic acid. In crude liver homogenates the accumulation of the free triacetic acid can be demonstrated with malonate, since this substance has been found to inhibit greatly the splitting of triacetic acid in such homogenates.

Triacetic Lactone Metabolism—As recorded previously, triacetic lactone is metabolized more slowly than the free acid by rat liver homogenate, with the formation of 1 mole of acetoacetic acid from 1 mole of triacetic acid. The latter point is in disagreement with the results of Breusch and Ulusoy (12) who found approximately 1.3 moles of acetoacetic acid formed from

---

[2] Dr. A. L. Dounce and coworkers in this laboratory have prepared a highly purified liver esterase.

1 mole of triacetic lactone. They concluded that this was sufficiently close to a theoretical 1.5 moles to support a β oxidation-recondensation type of metabolism for this compound. The reason for the difference in results obtained is not clear, but probably rests in the specificity and accuracy of the analytical methods employed.

In any event it is felt that the free acid is the more likely diketone metabolite if such compounds occur in fat metabolism, and this is supported by its much greater rate of metabolism. Furthermore the lactone of triacetic acid cannot be considered as being in simple chemical equilibrium with the free acid. This was pointed out in a previous paper (10) and is further emphasized by the finding that the purified triacetic acid enzyme does not affect the lactone. It seems probable that the formation of acetoacetic acid from triacetic lactone involves a preliminary formation of the free triacetic acid, but this point has not been thoroughly investigated. In this connection Breusch and Ulusoy found that the enzyme in liver homogenate which acts on the lactone was destroyed by heating for 5 minutes at 55°, a condition which we have found does not destroy the triacetic acid enzyme.

TABLE V

Metabolism of Ethyl Triacetate by Liver Enzymes

Incubation at 3.3 ml.; bicarbonate buffer, pH 7.4; temperature 30°; malonate 0.01 M.

Enzyme	Malonate	Time	Conversion of ester to	
			Triacetic acid	Acetoacetic acid
		min.	*per cent*	*per cent*
Liver homogenate	+	20	87	0
" "	–	20	58	37
" "	+	60	58	25
" "	–	60	0	90
Triacetic acid enzyme	–	55	0	0
Esterase	–	55	86	
" + triacetic acid enzyme	–	55		92

DISCUSSION

The data presented have demonstrated the cleavage of triacetic acid into acetoacetic and acetic acids by liver homogenates and by an enzyme isolated from liver, according to the equation $CH_3COCH_2COCH_2COOH \rightarrow CH_3COCH_2COOH + CH_3COOH$. This is the first demonstration of the biological activity of an open chain polyketonic acid which could be formed by "multiple alternate" β oxidation of a fatty acid. In conjunction with the fact that this postulated intermediate is metabolized at least as fast as the corresponding fatty acid, it would appear necessary to accommodate this type of compound in theories of fat metabolism.

In postulating triacetic acid as an intermediate of hexanoic acid oxidation, the acetoacetate yield from lower fatty acids demands consideration, since triacetic acid yields only 1 mole of acetoacetate. Either β or δ cleavage of triacetic acid is indicated with no recondensation of the resulting 2-carbon fragments. At least in the case of octanoic acid, β oxidation and cleavage with recondensation of 2-carbon units are indicated as the principal mode of acetoacetate production (9, 23). In the case of hexanoic acid, however, experiments on acetoacetate yield *in vitro* are somewhat at variance.

Leloir and Muñoz (24) and Jowett and Quastel (2) find the yield of acetoacetate from hexanoic acid greater than from butyric acid. On the other hand, calculations from Cohen's data (25) indicate the production of 1 mole of acetoacetate from hexanoic acid. The isotope experiments of Morehouse and Deuel (26) with hexanoic acid favor δ cleavage of a multiple "alternate oxidized" intermediate. It is of course possible that the mode of oxidation and cleavage of hexanoic and octanoic acids differs, and further experiments on the oxidation of hexanoic acid *in vitro* are in progress in this laboratory.

Successive condensation of acetic acid molecules of 2-carbon residues from carbohydrate catabolism is indicated in the biological synthesis of fatty acids (27). The pyruvate → acetoacetate reaction (28) and the acetate-acetoacetate conversion (21, 29) offer possibilities for the initial stages of fatty acid synthesis. Further stages are obscure, although the isotope experiments of Rittenberg, Schoenheimer, and Evans (30) do not favor butyric and hexanoic acids as intermediates in the synthesis. Further condensation of acetoacetate with acetate would yield triacetic acid, the intermediate under consideration in this paper. Thus polyketonic acids are implicated in fatty acid synthesis as well as catabolism.

SUMMARY

1. A method for the determination of acetoacetic acid in the presence of triacetic acid and related compounds has been described.

2. Triacetic acid is rapidly metabolized by rat liver homogenate and an enzyme isolated from liver, yielding in both cases 1 mole of acetoacetate and 1 of acetate.

3. Triacetic ester is rapidly metabolized by liver through the successive action of an esterase and the triacetic acid enzyme. Triacetic lactone is metabolized by rat liver at about one-eighth the rate of the free acid.

4. The implications of these findings to studies of fat metabolism are discussed.

BIBLIOGRAPHY

1. Hurtley, W. H., *Quart. J. Med.*, **9**, 301 (1916).
2. Jowett, M., and Quastel, J. H., *Biochem. J.*, **29**, 2159 (1935).
3. Stadie, W. C., *Physiol. Rev.*, **25**, 395 (1945).
4. Stotz, E., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **5**, 129 (1945).
5. Krebs, H. A., and Johnson, W. A., *Biochem. J.*, **31**, 645 (1937).
6. Lehninger, A. L., *J. Biol. Chem.*, **148**, 393 (1943).
7. Meister, A., and Greenstein, J. P., *Federation Proc.*, **7**, 173 (1948).
8. Lehninger, A. L., *J. Biol. Chem.*, **153**, 561 (1944).
9. Weinhouse, S., Medes, G., and Floyd, N. F., *J. Biol. Chem.*, **155**, 143 (1944).
10. Witter, R. F., and Stotz, E., *J. Biol. Chem.*, **176**, 485 (1948).
11. Witter, R. F., Snyder, J., and Stotz, E., *J. Biol. Chem.*, **176**, 493 (1948).
12. Breusch, F. L., and Ulusoy, E., *Arch. Biochem.*, **14**, 183 (1947).
13. Alyea, H. N., and Backström, H. J., *J. Am. Chem. Soc.*, **51**, 90 (1929).
14. Coombes, A., *Ann. chim. et phys.*, **12**, 229 (1887).
15. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **39**, 408 (1945).
16. Barnes, R. H., and Wick, A. H., *J. Biol. Chem.*, **131**, 413 (1939).
17. Dounce, A. L., and Beyer, G. T., *J. Biol. Chem.*, **174**, 859 (1948).
18. Lehninger, A. L., *J. Biol. Chem.*, **157**, 363 (1945).
19. Friedemann, T. E., *J. Biol. Chem.*, **123**, 161 (1938).
20. Stadie, W. C., Zapp, J. A., Jr., and Lukens, F. D. W., *J. Biol. Chem.*, **137**, 75 (1941).
21. Lehninger, A. L., *J. Biol. Chem.*, **143**, 147 (1942).
22. Krüger, D., and Tschirch, E., *Ber. chem. Ges.*, **62**, 2776 (1929); **63**, 826 (1930).
23. Buchanan, J. L., Sakami, W., and Gurin, S., *J. Biol. Chem.*, **169**, 411 (1947).
24. Leloir, J. M., and Muñoz, L. F., *Biochem. J.*, **33**, 734 (1939).
25. Cohen, P. P., *J. Biol. Chem.*, **119**, 333 (1937).
26. Morehouse, M. G., and Deuel, H. J., *Proc. Soc. Exp. Biol. and Med.*, **45**, 96 (1941).
27. Rittenberg, D., and Bloch, K., *J. Biol. Chem.*, **154**, 311 (1944).
28. Lehninger, A. L., *J. Biol. Chem.*, **164**, 291 (1946).
29. Medes, G., Floyd, N. F., and Weinhouse, S., *J. Biol. Chem.*, **162**, 1 (1946).
30. Rittenberg, D., Schoenheimer, R., and Evans, E. A., Jr., *J. Biol. Chem.*, **120**, 503 (1937).

STUDIES ON NITROGEN METABOLISM IN TOMATO WITH USE OF ISOTOPICALLY LABELED AMMONIUM SULFATE*

By ROBERT MacVICAR† and R. H. BURRIS

(*From the Department of Biochemistry, University of Wisconsin, Madison*)

(Received for publication, July 16, 1948)

The stable isotope of nitrogen, N^{15}, has been employed extensively as a tracer in studies of nitrogen transformations in animals and bacteria, and its use has yielded much information difficult or impossible to obtain otherwise. The extension of this method to studies involving the higher plants, however, has been very limited. Hevesy and coworkers (1) studied the absorption of ammonium ions labeled with N^{15} in the sunflower and showed that there was a definite replacement of the nitrogen present in the mature leaf. Vickery and others (2) employed tobacco and buckwheat and found at the end of 72 and 47 hours, respectively, incorporation of N^{15} from $N^{15}H_4Cl$ into the principal nitrogenous fractions of the plants.

The absorption, translocation, and utilization of inorganic nitrogen by plants has been extensively investigated by means of traditional methods. The demonstration in animals of a rapid and continuous interchange between the various forms of nitrogen (3), however, raises serious doubt concerning the interpretation of some of these earlier findings. Studies of the metabolic pathways for the incorporation of inorganic nitrogen into tissue protein and other nitrogenous constituents of higher plants have therefore been begun with the stable isotope of nitrogen as a tracer. This communication presents certain limited observations of nitrogen metabolism in tomato plants.

EXPERIMENTAL

Materials and Methods

Tomato plants (*Lycopersicon esculentum* Miller, var. John Baer) were used throughout this study. Young seedlings, sprouted in soil, were transferred to silica sand in 10 inch varnished clay pots. A complete nutrient solution (4) containing both ammonium and nitrate nitrogen was supplied. The plants were grown under normal greenhouse conditions of light and temperature, the latter ranging from 24–29° during the day and held near 18° during the night.

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

† Present address, Department of Agricultural Chemistry Research, Oklahoma Agricultural Experiment Station, Stillwater.

Experiment I—Seedlings 6 weeks old were employed in this study. 14 days before the administration of the labeled isotope, the pots were thoroughly leached and during the ensuing period supplied a nutrient solution without nitrogen. At the end of this period of deprivation, distinct symptoms of nitrogen deficiency had appeared. At 9.00 a.m. 15 mg. of ammonium nitrogen as $(N^{15}H_4)_2SO_4$ containing 30 atom per cent N^{15} excess were supplied each pot. The drain was closed with a stopper and distilled water added to saturation. The greenhouse temperature was near 27° and transpiration was rapid. 4 hours later the plants were harvested, separated into tops and roots, and the latter thoroughly washed in tap water to remove adhering salts. The plant parts were rapidly heated in a steam-jacketed oven to approximately 65° and then transferred to a forced draft oven maintained at 60° for 8 hours. After an additional 18 hours in an ordinary 60° oven, the tissues were weighed, the adhering sand removed from the roots, and the parts ground in a mortar to pass a 40 mesh sieve. In this manner 10.5 gm. of dry leaf plus stem tissue and 4.1 gm. of dry root tissue were obtained. Nitrogen fractionation was performed upon these dry materials.

Experiment II—This experiment was essentially a repetition of Experiment I, but slightly larger seedlings, about 2 months old, were used. Nitrogen deficiency was induced by omission of nitrogen from the nutrient solution from March 15 to 27, 1946. On the last day 15 mg. of ammonium nitrogen as $(N^{15}H_4)_2SO_4$ containing 30 atom per cent N^{15} excess were supplied at 7.00 a.m. After 12 hours of conditions conducive to a high transpiration rate (temperature, 24–27°, light intensity, 850 to 950 foot candles at the bench surface) the plants were harvested, heated, and dried as before. The tissues were ground in a Wiley mill and stored for analysis.

Nitrogen Fractionation Procedures—The nitrogen fractionation procedures employed were essentially those of Vickery and coworkers (5), and consisted of ethanol extraction in a continuous drip extractor for 12 hours (ethanol-soluble fraction) followed by repeated extraction with small portions of boiling water, centrifugation, and filtration through a small plug of glass wool (water-soluble fraction). The hot water-insoluble nitrogen has been shown to consist almost wholly of proteins (protein fraction). This latter material was then hydrolyzed with 8 N H_2SO_4 under a reflux for 24 hours, the humin filtered off (humin nitrogen), the sulfate ions removed with $Ba(OH)_2$, and the free amino acids precipitated at approximately pH 8 by mercuric acetate followed by the addition of alcohol to a final concentration of 30 per cent. The insoluble material (Neuberg precipitate) was separated from the liquid (Neuberg filtrate) by centrifugation. Mercury was removed from the acidified Neuberg precipitate as the sulfide; the basic amino acids were precipitated from the resulting solution by the addition of phosphotungstic acid (PTA precipitate and PTA filtrate). In Experiment II, following the precipitation of the amino acids and similar compounds by the Neuberg procedure, various amino acids or amino acid groups were isolated. The methods employed were essentially those described by Schoenheimer, Ratner, and Rittenberg (6).

Measurement of Isotope Concentration—The concentration of N^{15} in the various fractions and compounds was determined by Kjeldahl digestion, distillation of NH_3 from alkaline solution, and subsequent conversion to N_2 by alkaline hypobromite. Measurement of isotope concentration in the N_2 was made with a mass spectrometer of the Nier type; the error of the determinations was less than ±0.05 atom per cent. Isotope concentration is expressed as atom per cent N^{15} excess.

TABLE I

Isotope Concentration of Various Nitrogen Fractions of Tomato Leaf, Stem, and Root Tissue Supplied $(N^{15}H_4)_2SO_4$ for 4 Hours

The results are expressed as atom per cent N^{15} excess.

Nitrogen fraction	Isotope concentration	
	Root tissue	Leaf and stem tissue
Total nitrogen	2.42	0.33
Water-soluble	7.09	0.71
Ammonia	12.06	1.15
Amide	2.17	0.10
Remaining water-soluble	1.89	0.63
Humin of hydrolysate	0.45	0.06
Neuberg filtrate	0.18	0.25
" ppt	0.27	0.08
PTA filtrate	0.24	0.08
" ppt	0.31	0.08

Results

The isotope concentrations of various nitrogenous fractions of the rapidly growing tomato plants from Experiment I are presented in Table I. Examination of the data reveals that during the 4 hour period rapid assimilation of the labeled ammonium ion occurred; the total nitrogen of root and top attained values of 2.42 and 0.33 atom per cent N^{15} excess, respectively. The ammonia fraction of root tissue contained 12.06 atom per cent N^{15} excess; thus, the absorbed ammonia nitrogen represented some 40 per cent of the total ammonia nitrogen present in the roots after 4 hours.

The data obtained in Experiment II are presented in Table II. It will be noted that in these larger, more mature plants the concentration of isotopic nitrogen attained in 12 hours in the roots was considerably less than that attained in the roots of the younger plants (Experiment I) in 4 hours.

However, the 12 hour period permitted more extensive translocation and assimilation of N^{15} in the tops than did the 4 hour treatment. Considerable difficulty was experienced in isolating the amino acids from the small amount of protein from the root tissue. Several additional amino acids were obtained, but not in sufficient yield to permit satisfactory purification. Likewise in the leaf tissue protein hydrolysates, the small quantity of total amino acid nitrogen did not permit a separation of the individual amino acids present in the copper salts fractions. According to Town (7) the principal amino acids found in the various copper salt fractions are as follows: water-insoluble copper salts, leucine, phenylalanine, cystine, methionine, and any tyrosine or aspartic acid not previously removed; methyl alcohol-soluble copper salts, proline, hydroxyproline, isoleucine, and valine.

TABLE II

Isotope Concentration of Amino Acids and Amino Acid Fractions of Tomato Leaf, Stem, and Root Tissue Supplied $(N^{15}H_4)_2SO_4$ for 12 Hours

The results are expressed as atom per cent N^{15} excess.

Fraction	Isotope concentration	
	Root tissue	Leaf and stem tissue
Total nitrogen	1.13	1.02
Ethanol-soluble	3.89	1.74
Water-soluble	0.77	0.49
Protein fractions		
Hydrolysate	0.79	0.58
Humin	0.45	0.21
Neuberg ppt	0.56	0.64
" filtrate	0.68	0.25
NH_3 + amide	0.38	0.72
PTA ppt		0.56
" filtrate		0.82
Water-insoluble copper salts		0.71
Methanol-soluble " "		0.84
Arginine		0.77
Glutamic acid	1.15	4.16
Aspartic "	0.75	0.94
Histidine		0.21
Lysine		0.07

DISCUSSION

The speed with which rapidly growing seedlings absorb, translocate, and utilize inorganic nitrogen is clearly shown from the data of Experiment I. During a period of 4 hours, the amount of nitrogen taken into and retained by tomato roots was roughly 8 per cent of the total assimilated by the roots in their entire growth. During the same period the tops accumulated N^{15}-labeled nitrogen equivalent to about 1.1 per cent of their total nitrogen. This rapid absorption was probably accentuated by the nitrogen-deficient state of the plants. Conditions for rapid translocation also existed during this period and likely promoted rapid upward carriage of the absorbed ions.

Subsequent to the addition of N^{15}-enriched ammonia the N^{15} was incorporated into amino acids and tissue proteins. The data do not reveal how much of the N^{15} of the proteins was incorporated by total synthesis of the proteins and how much by exchange of amino acids with the amino acids of preformed proteins. Although the total nitrogen assimilated in the tops was quantitatively greater than in the roots, the percentage of most nitrogenous fractions formed during the period of treatment was greater in the roots, as attested by their higher atom per cent N^{15} excess.

Glutamine and asparagine, produced in quantity by certain plants during their early growth, are depleted as the plants mature. The amide nitrogen apparently is used in the synthesis of amino acids and proteins in the maturing plant. The young plants of Experiment I accumulated a rather high concentration of ammonia in their roots and in response to this abundant supply of ammonia synthesized amides in considerable amounts. The lower supply of ammonia to the tops was reflected in a much less vigorous amide synthesis there. The older plants of Experiment II accumulated less ammonia and synthesized a lower concentration of amides (analyzed in the protein fraction) than did the young plants, but during the 12 hour period of treatment there was a higher percentage increase of amides in their tops than in their roots from the ammonia supplied. The N^{15} concentration of the amide fraction in the roots of the young plants (Experiment I) is high relative to the other fractions, but this was not true for the amides elsewhere. Apparently their importance was largely confined to young root tissue high in ammonia nitrogen. The amide fractions are not as clear cut as might be desired, for the treatment of the tissue was such that partial hydrolysis may have occurred; the amide and ammonia fractions in Experiment II were not separated and were determined in the protein fractions only.

The dicarboxylic amino acids, glutamic and aspartic, contained high concentrations of N^{15}, indicating their rapid synthesis or turn over. In the leaf and stem tissue protein (Table II) glutamic acid was found to have an N^{15} concentration 7 times the average for the whole protein. A similar high order of reactivity of these two amino acids has been observed in the tissues of rats fed isotopically labeled ammonium citrate (8) and in tobacco plants furnished the ammonium ion (2). These data also show a lower order of reactivity for aspartic acid in tomato, of interest in view of the dominance of glutamine over asparagine in this species (9). The probable importance of oxalacetate and α-ketoglutarate in carbohydrate transformations in the higher plants is now generally recognized. The rapid formation from ammonium nitrogen of the corresponding amino acids supports the suggestions of Chibnall (10) and Vickery and Pucher (11) concerning the importance of these α-keto acids in nitrogen metabolism.

Histidine was found to contain relatively less N^{15} (0.21 atom per cent N^{15} excess) than most of the other amino acids or fractions. Lysine showed a particularly low order of reactivity as indicated by an isotope content of only 0.07 atom per cent excess. An intermediate concentration of N^{15} was found in arginine, indicating that this amino acid is relatively more active metabolically than the other basic amino acids. The isotope concentration of the phosphotungstic acid filtrate, containing principally the monoamino monocarboxylic amino acids, was also intermediate. Two copper salt fractions were isolated and similar isotope concentrations found in each.

SUMMARY

Tomato plants were supplied for a short time with $(NH_4)_2SO_4$ labeled with N^{15}. Various nitrogenous fractions and amino acids were then isolated and analyzed for their concentration of N^{15}. Rapid absorption, translocation, and utilization of the ammonium ion were found to have occurred after 4 and 12 hours. All nitrogenous fractions or amino acids isolated contained significant excesses of N^{15}. Among the amino acids and amino acid fractions separated, glutamic acid had an outstandingly high concentration of N^{15}; aspartic acid also had a high level of N^{15}. In young plants the distribution of N^{15} indicated a considerable synthesis of amides, particularly in the roots where much ammonia was present. Of the compounds isolated the basic amino acids, histidine and lysine, had the lowest N^{15} concentration.

BIBLIOGRAPHY

1. Hevesy, G., Linderstrøm-Lang, K., Keston, A. S., and Olsen, C., *Compt. -rend. trav. Lab. Carlsberg, Série chim.*, **23**, 213 (1940).
2. Vickery, H. B., Pucher, G. W., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **135**, 531 (1940).
3. Schoenheimer, R., The dynamic state of body constituents, Cambridge (1942).
4. MacVicar, R., and Struckmeyer, B. E., *Bot. Gaz.*, **107**, 454 (1946).
5. Vickery, H. B., Pucher, G. W., Leavenworth, C. S., and Wakeman, A. J., *Connecticut Agr. Exp. Sta., Bull. 374*, 557 (1935).
6. Schoenheimer, R., Ratner, S., and Rittenberg, D., *J. Biol. Chem.*, **130**, 703 (1939).
7. Town, B. W., *Biochem. J.*, **30**, 1837 (1936).
8. Foster, G. L., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **127**, 319 (1939).
9. Vickery, H. B., Pucher, G. W., and Clark, H. E., *Science*, **80**, 459 (1934).
10. Chibnall, A. C., Protein metabolism in the plant, New Haven (1939).
11. Vickery, H. B., and Pucher, G. W., *J. Biol. Chem.*, **128**, 703 (1939).

ANTAGONISM OF AMINO ACIDS IN THE GROWTH OF LACTIC ACID BACTERIA*

BY W. L. BRICKSON, L. M. HENDERSON, I. SOLHJELL,† AND C. A. ELVEHJEM

(*From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*)

(Received for publication, July 16, 1948)

Inhibition of microbial growth by compounds closely related to essential metabolites has been demonstrated by many investigators (1–3). Early workers (4–7) showed that inhibitory effects of single amino acids could be reversed by altering the concentration of structurally related amino acids. Gladstone (8), using *Bacillus anthracis*, showed such a relationship among leucine, valine, and isoleucine and between threonine and serine. Threonine exerts an antagonistic action on the utilization of serine by *Lactobacillus arabinosus* (9). Using *Lactobacillus casei*, Feeney and Strong (10) demonstrated an inhibitory effect of aspartic acid, reversible by glutamine, glutamic acid, or asparagine.

While investigating a uniform medium for the microbiological determination of amino acids (11), certain difficulties were encountered with the glutamic acid and isoleucine assays with *Lactobacillus arabinosus*. A lag in the growth response curve was observed at the lower concentrations of these amino acids. The tubes containing samples, particularly in the case of isoleucine assays, did not show this lag to the same extent as the standard curves, resulting in marked downward drift of assay values. To alleviate the lag in the glutamic acid curve and to give a valid assay, a heavy inoculum and adjustment of the medium to pH 6 have been used (12). While this provided a satisfactory assay for glutamic acid, further work on the fundamental defect was indicated. As in previous studies (13, 12, 14, 15), when glutamic acid standard was replaced by glutamine (sterilized by filtration) the lag was absent. This indicated, as suggested previously (12, 14), that glutamic acid is utilized through glutamine and that the lag is the result of partial or complete failure of the small amounts of glutamic acid present to be amidated to glutamine.

In this study the mutual antagonism of the members of two groups of

---

* Published with the approval of the Director of the Wisconsin Alumni Research Foundation. Supported in part by grants from the Abbott Laboratories, North Chicago, Illinois, the Nutrition Foundation, Inc., New York, and the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

† Present address, Oslo, Norway.

amino acids in the growth of *Lactobacillus arabinosus* 17-5 and *Leuconostoc mesenteroides* P-60 was investigated.

EXPERIMENTAL

The cultures and assay techniques used were the same as described previously (11), except that light inocula were used. To accentuate the lag in growth, the inocula were diluted until no turbidity was perceptible. In later experiments, the inocula were carefully standardized to a somewhat greater dilution than this by suspending the cells in sufficient sterile water to give a reading of 50 in an Evelyn colorimeter, with standard Evelyn tubes, and a 660 mμ filter against a water blank. 1 ml. of this suspension was then diluted to 200 ml. with sterile, distilled water and 1 drop was used to inoculate each 2 ml. of culture medium.

In most studies, 2 ml. volumes in 18 × 150 mm. culture tubes were used. In some of the glutamic acid experiments early growth in 10 ml. volumes, in Evelyn colorimeter tubes, was measured turbidimetrically against an uninoculated blank. In other work where a number of solutions had to be sterilized by filtration 0.2 ml. volumes were used. They were titrated electrometrically (16) after 60 to 72 hours of incubation.

Results

Effect of Other Amino Acids on Isoleucine Requirement—Inhibition indices (3) were determined for *Lactobacillus arabinosus* and *Leuconostoc mesenteroides* with isoleucine as the metabolite and leucine and valine as the antagonists. Typical results are shown in Table I. The inhibition indices were not constant, but were of the same general magnitude over wide concentration ranges. In all cases increasing the concentration of the metabolite reversed the inhibition, indicating that it is competitive. DL-Valine and L-leucine were additive in antagonizing the growth of *Lactobacillus arabinosus* when isoleucine was the limiting amino acid. DL-Leucine was approximately half as effective as L-leucine as an antagonist of isoleucine, indicating that only the L isomer is involved; D-leucine was not tested. Decreasing the concentration of DL-valine and L-leucine in the basal medium from the normal level of 400 and 200 γ per 2 ml. tube to 100 and 50 γ, respectively, eliminated the lag.

Excessive amounts of methionine inhibited the growth of *Lactobacillus arabinosus* when isoleucine was the limiting amino acid. 10 mg. per tube of DL-methionine completely suppressed the growth of *Lactobacillus arabinosus* in the presence of 30 γ of DL-isoleucine. Alanine at 30 mg., or DL-serine or DL-threonine at 50 mg. per tube, caused no inhibition, indicating that the antagonism is not general for all amino acids (Table II). That these relationships are not peculiar to the medium being used in these studies was indicated by parallel experiments in which a medium typical of those currently employed by many other workers (17) was used.

TABLE I

Results of Typical Experiment Showing Effect of Valine and Leucine As Antagonists of Isoleucine for Lactobacillus arabinosus

DL-Isoleucine	DL-Valine	L-Leucine	Titer	Molar inhibition index*
γ per tube	*γ per tube*	*γ per tube*		
30	500	50	82	
30	1,000	50	72	
30	1,500	50	54	
30	2,000	50	6	45–60
200	15,000	50	192	
200	20,000	50	181	
200	25,000	50	102	
200	30,000	50	67	
200	45,000	50	13	
2000	15,000	50	195	
2000	75,000	50	179	135–200
6	100	100	9	
6	100	500	5	
30	100	200	105	
30	100	400	87	
30	100	800	53	
30	100	1000	14	27–33

* Based on concentrations of L forms of amino acids.

TABLE II

*Summary of Results of Studies of Antagonism of Amino Acids**

Limiting amino acid	Inhibiting amino acid	Molar inhibition index	
		L. arabinosus	*L. mesenteroides*
Isoleucine	DL-Valine	45–60	500–1000
	L-Leucine	30–40	75–250
	DL-Leucine	40–50	Not determined
	DL-Methionine	100–200	" "
	DL-Serine	None at 4500	" "
	DL-Threonine	" " 3500	" "
	DL-Alanine	" " 1400	" "
Leucine	DL-Isoleucine	450	None at 3000
	DL-Valine	2000	
	DL-Methionine	None at 1500	Not determined
	DL-Threonine	" " 5500	" "
	DL-Serine	" " 6200	" "
Valine	L-Leucine	360	None at 1800
	DL-Isoleucine	20–40	700
	DL-Methionine	<300	Not determined

* All based on concentrations of the L isomers.

When five organisms were used to determine isoleucine in acid-hydrolyzed casein, all except *Lactobacillus arabinosus* gave nearly identical values, with no drift. With *L. arabinosus* high values at the lower levels and low values in the upper part of the standard curve, *i.e.* a drift downward, were encountered. Thus, for example, values of 6.1 per cent with *Streptococcus faecalis* R, 6.0 per cent with *Leuconostoc mesenteroides*, 6.0 per cent with *L. delbrueckii*-3, and 6.1 per cent with *L. casei* were obtained. Values with *L. arabinosus* drifted from 10 to 5.6 per cent, indicating that the standard curve was below the "sample curve" at low levels and above it at the higher levels.

TABLE III

*Effect of Regular and Low Leucine and Valine Concentrations in Medium on Isoleucine Assay of Casein Hydrolysate**

Sample weight	*L. mesenteroides*		*L. arabinosus*	
	Low	Regular	Low	Regular
mg.				
0.1	6.4	9.5	6.5	15.7
0.2	6.8	7.3	4.6	9.3
0.3	6.0	6.4	3.8	7.4
0.4	6.0	6.2	3.7	6.2
0.5	6.3	6.0	3.3	5.7
Average.........	6.3	7.1	4.4	8.9

* Regular levels were DL-valine 0.4 mg. per 2 ml. tube and L-leucine 0.2 mg. per 2 ml. tube. Low levels were one-fourth of the regular concentrations.

The effect of lowering the level of leucine and valine in the medium for the determination of isoleucine in another protein hydrolysate is shown in Table III. With regular concentrations of leucine and valine in the medium, there was drift in the values, particularly with *Lactobacillus arabinosus*. Decreasing the level of these amino acids eliminated the slight drift for *Leuconostoc mesenteroides*, giving assay values which compare well with those obtained with *L. delbrueckii*-3 (6.6 per cent). When *L. arabinosus* was the test organism, the drift was not eliminated by lowering the concentrations of leucine and valine in the medium, and much lower values were obtained. With *Leuconostoc mesenteroides*, inhibition of growth occurred in the lower portion of the curve when regular levels of leucine and valine were present in the medium, but the upper portion of the curve coincided with that obtained with low levels of these two amino acids (Curves 2 and 3, Fig. 1). With *L. arabinosus*, low levels of leucine and valine gave a standard curve elevated above that obtained with a medium containing regular concentrations of leucine and valine, at all levels of isoleucine (Curves 1 and 4, Fig. 1). This indicated utilization of the D isomer of isoleucine by *L. arabinosus* at low levels of leucine and valine. To verify the variable utilization of D-isoleucine by this organism, when regular and low levels of leucine and valine were used, standard curves with L- and DL-isoleucine were prepared. With regular levels of leucine and valine the L and DL standard curves (Curves 1 and 2, Fig. 2) nearly coincided, except in the upper portion. In this portion, the ratio of the concentrations of leucine and valine to isoleucine was less than in the lower portion of the curve, resulting in greater utilization of the D isomer. With low levels of leucine and valine present in the medium, the L and DL curves were not superimposable. The DL-isoleucine standard curve (Curve 4, Fig. 2) diverged upward from the L-isoleucine standard curve (Curve 3, Fig. 2), indicating that the D isomer was being utilized at nearly all concentrations of isoleucine. When D-isoleucine was added in increasing concentrations to a medium containing the L isomer at a level sufficient for half maximum growth (7.5 γ per tube), the titration values increased, indicating 10 per

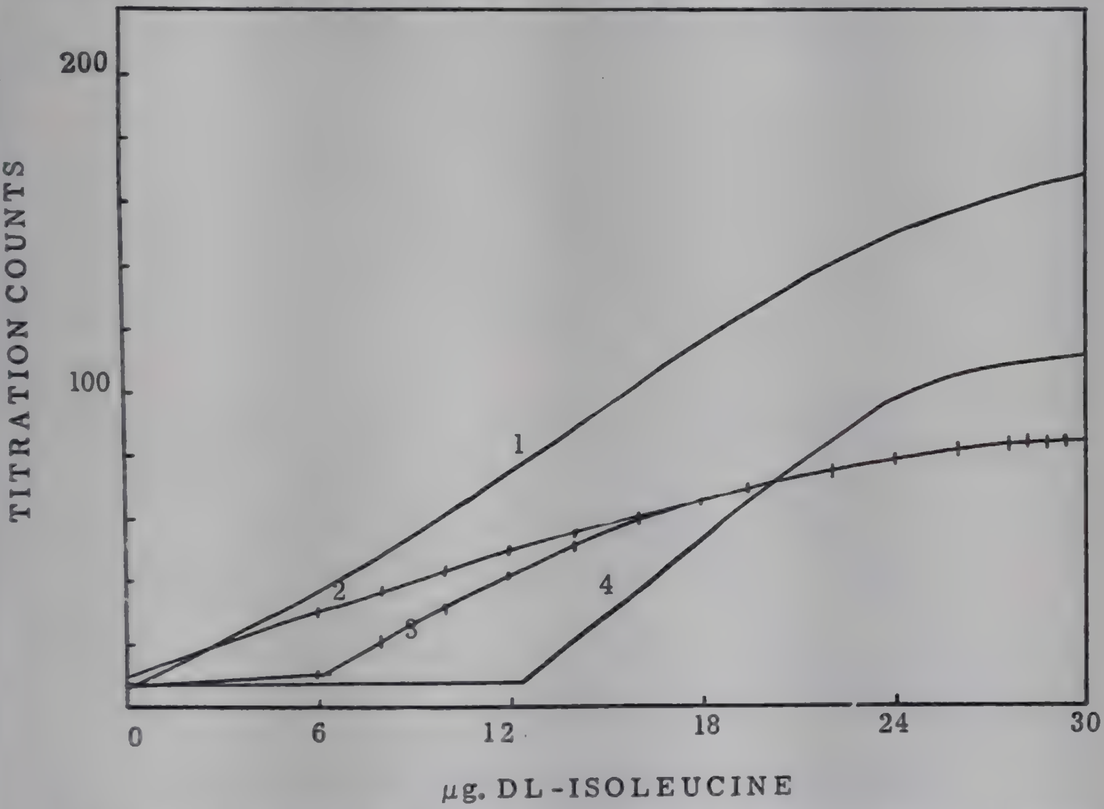


FIG. 1. Effect of leucine and valine concentrations on isoleucine standard curves. Curves 1 and 2, L-leucine 0.05 mg. and DL-valine 0.1 mg. per 2 ml. tube; Curves 3 and 4, regular levels, L-leucine 0.2 mg. and DL-valine 0.4 mg. per 2 ml. tube Curves 1 and 4, *Lactobacillus arabinosus*; Curves 2 and 3, *Leuconostoc mesenteroides*.

cent activity at 10 γ per tube, and 19 per cent activity at 30 γ per tube. When D-isoleucine was used alone for preparing a standard curve, it showed no growth-promoting activity.

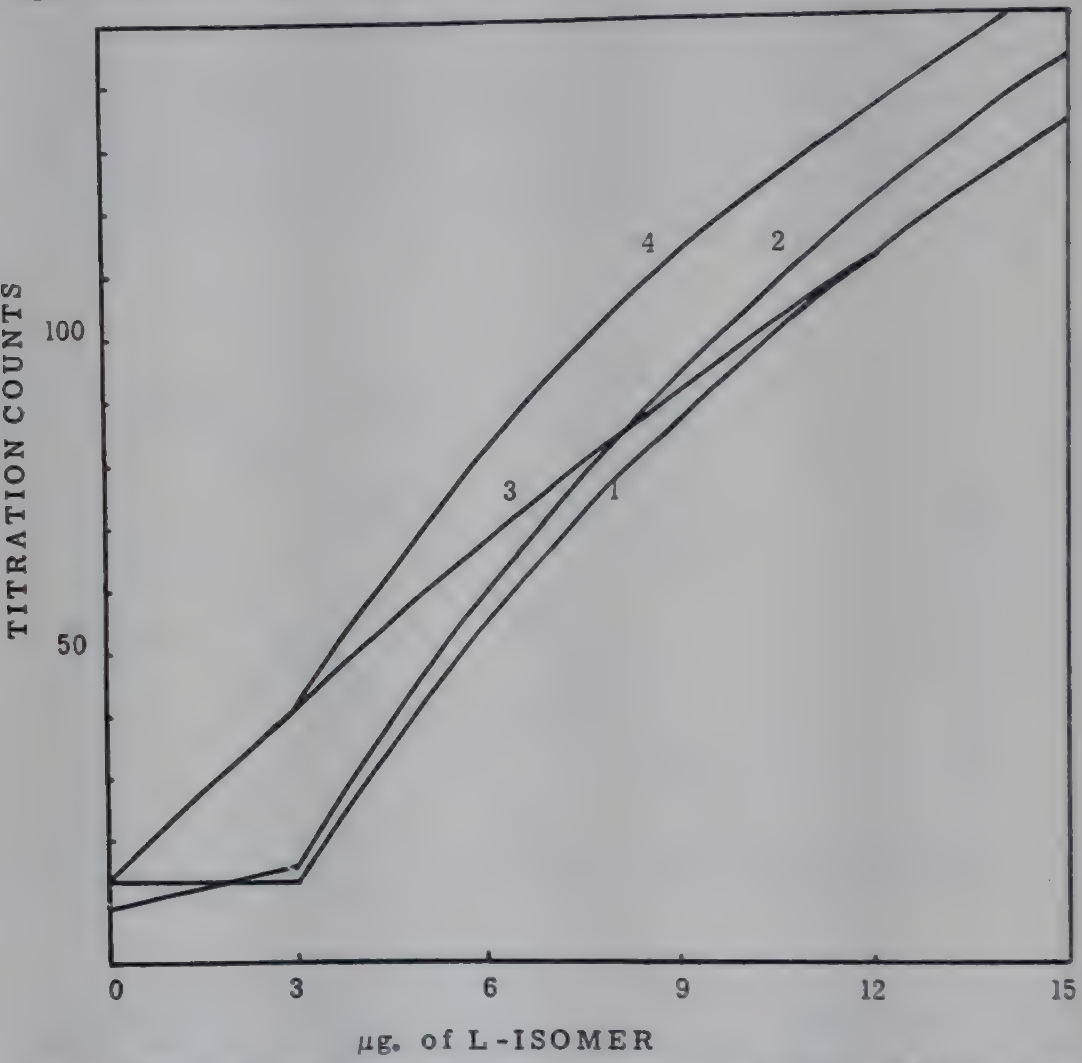


FIG. 2. Effect of regular and low leucine and valine on response of *Lactobacillus arabinosus* to L- and DL-isoleucine. Curve 1, L-isoleucine, regular leucine and valine; Curve 2, DL-isoleucine, regular leucine and valine; Curve 3, L-isoleucine, low leucine and valine; and Curve 4, DL-isoleucine, low leucine and valine.

During these studies the contamination of DL-isoleucine (Merck), presumably with alloisoleucine, was noted. The activity for *Leuconostoc mesenteroides* and *L. delbrueckii*-3 of this DL-isoleucine, as compared to a pure sample of L-isoleucine,[1] was only 39.5 per cent, instead of the 50 per cent expected for pure DL-isoleucine. This was also observed by Mr. F. A. Wacher of Merck and Company, Inc., and was reported by Smith and Greene (18).

---

[1] Kindly supplied by Dr. D. G. Doherty of this laboratory.

Effect Of Other Amino Acids on Leucine and Valine Requirements—With leucine limiting, the concentrations of either DL-valine or DL-isoleucine required to inhibit growth of *Lactobacillus arabinosus* were nearly the same. The inhibition indices (Table II) indicate that this organism is less sensitive to these antagonisms when leucine is limiting than when isoleucine is the limiting nutrient. Methionine, threonine, and serine did not show the antagonism at the high concentrations used.

When valine was the limiting amino acid for the growth of this organism, DL-isoleucine caused inhibition at lower concentrations than did L-leucine. From Table II it is evident that leucine is antagonized less by isoleucine and valine than is valine by isoleucine and leucine. These differences account for the occasional slight lag in the valine standard curves with this medium and this organism and the absence of such lags in the leucine curves with similar assay conditions. The presence or absence of a lag from one valine assay to another is probably a result of slight variations in the weight and age of the inoculum. Methionine antagonized the utilization of limiting quantities of valine.

An antagonizing action by isoleucine for *Leuconostoc mesenteroides* with low concentrations of leucine could not be shown. Growth could be inhibited, however, by large amounts of isoleucine when the valine level was limiting. The relatively lower susceptibility of *Leuconostoc mesenteroides* to these imbalances is probably one reason why this organism is now so widely used for amino acid assays.

Glutamic Acid-Aspartic Acid Relationship—The general lag in the growth of *Lactobacillus arabinosus* when glutamic acid was limiting was evidenced in the lower portion of the standard curve, but occasionally extended over one-half of the range. Adjustment of the medium to pH 6 together with the use of a heavy inoculum (12) was a practical solution. Lyman *et al.* (14) added small amounts of glutamine to prevent this lag. Replacement of aspartic acid by asparagine has been reported by Baumgarten *et al.* (15) to relieve the lag, but when asparagine was added to our medium already containing aspartic acid, the lag was accentuated. This suggested that aspartic acid or asparagine might be the active substance causing the lag in the growth curve. When the concentration of aspartic acid in the medium was progressively lowered, the lag was diminished until it was completely eliminated at 40 γ per tube (Fig. 3). A 10- to 20-fold greater concentration of asparagine than aspartic acid was necessary to elicit an equivalent lag in growth. Table IV shows the effect of asparagine and aspartic acid on the metabolism of glutamic acid by *L. arabinosus*. These data show an inhibition index of 400 for asparagine and approximately 20 for L-aspartic acid. Asparagine exerts a stimulatory effect on the growth of this organism (Table IV) as shown by slightly higher titrations

TABLE IV

*Effect of Asparagine and Aspartic Acid on Metabolism of Glutamic Acid for Lactobacillus arabinosus**

Concentration of glutamic acid	No aspartic acid	Concentration			
		L-Asparagine, 2 mg.	DL-Asparagine, 2 mg.	L-Aspartic acid, 2 mg.	DL-Aspartic acid, 2 mg.
γ					
0	3	3	4	5	5
5	20	7	4	5	4
10	30	25	20	5	5
25	43	56	56	5	5
80	86	102	98	5	5
100	96	108	101	11	72

* Final pH of media after autoclaving 7.1. Each count represents 0.05 ml. of 0.04 N NaOH.

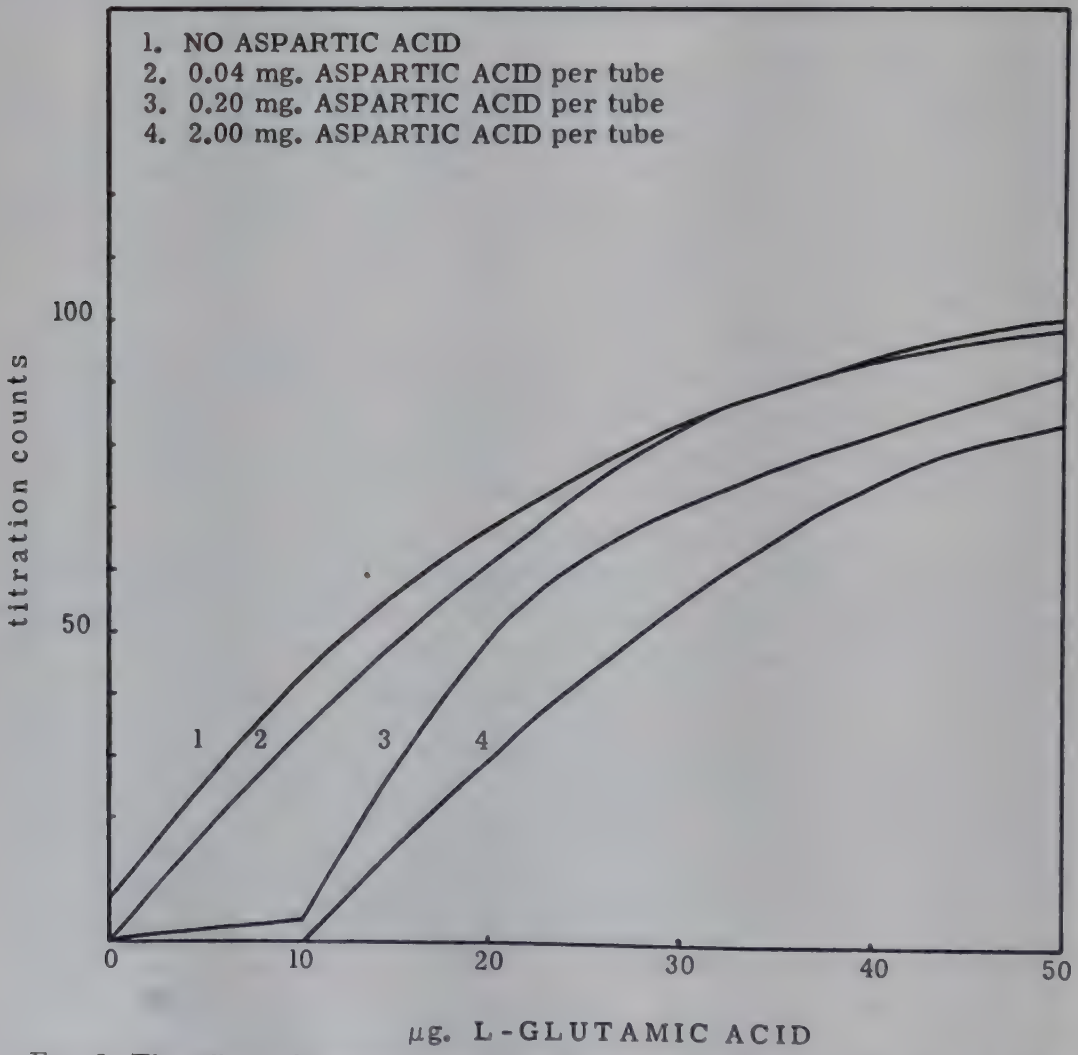


FIG. 3. The effect of L-aspartic acid and L-asparagine concentration on the response of *Lactobacillus arabinosus* to glutamic acid.

for the medium containing added asparagine. Three different samples of asparagine (one of racemate and two of the L form) were used, at the same concentration, to test the possibility that the lag was due to aspartic acid as a contaminant of the asparagine. All samples gave the same degree of lag in growth. It is unlikely that three different samples would contain equal amounts of aspartic acid. From these data, it appears that the antagonistic action is due to the asparagine *per se*.

With aspartic acid limiting, high glutamic acid concentrations had no effect on the growth of *Leuconostoc mesenteroides*.

TABLE V

Inhibition of Growth of Lactobacillus arabinosus by Bicarbonate and L-Aspartic Acid in Presence of 0.425 × 10^{-3} M Glutamic Acid or Glutamine

$NaHCO_3$	L-Aspartic acid	Glutamic acid*	Molar inhibition index	Glutamine*	Molar inhibition index
0	0	137		120	
10.9×10^{-3}	0	133		114	
23.8×10^{-3}	0	22	56	35	56
35.6×10^{-3}	0	2	83	1	83
47.6×10^{-3}	0	0		0	
59.5×10^{-3}	0	0		0	
0	0	147		130	
0	7.5×10^{-3}	21	19	135	
0	15.0×10^{-3}	8	35	135	
0	22.5×10^{-3}	8		136	
0	30.0×10^{-3}	7		136	
0	37.7×10^{-3}	7		137	

* The values represent titration counts, each count equivalent to 0.05 ml. of 0.04 N NaOH.

During these studies, Waelsch *et al.* (13) reported inhibition of the growth of *L. arabinosus* by oxalacetate and sodium bicarbonate. To determine whether these antagonisms had a common basis, inhibition indices were determined for L-aspartic acid and sodium bicarbonate. Table V shows the results of one such experiment. Although inhibition by aspartic acid was not observed when glutamine was present, glutamine had no effect on the bicarbonate inhibition. The molar inhibition index for aspartic acid was considerably lower than that for sodium bicarbonate.

DISCUSSION

The amino acid requirements of many lactic acid organisms have been studied extensively in the search for more specific and reliable assays, but little has been reported on the effect of high concentrations of amino acids.

The policy has been to add an excess of all required nutrients, with the exception of the one being assayed. The results reported here and those of Meinke and Holland (9) indicate that the amino acids should also be present in correct proportions to avoid imbalances. Addition of an inhibiting amino acid with the sample might result in growth suppression in the sample tubes not encountered in the standard tubes, with resulting drift and invalidation of the assay. This might dictate a medium containing little more of such amino acids than the bacteria need for maximum growth. When such a medium is used, however, the percentage difference in concentration in sample tubes and standard tubes is very much greater and might prove quite significant when proteins of other than average composition are assayed. A safer procedure appears to be that of maintaining moderately high concentrations of all constituents, so that the percentage change in composition of the fermentation liquid is affected only slightly by addition of the sample. In cases in which difficulties arise, as evidenced by lag, drift in assay values, poor recovery of added amino acid, or inconsistent values, other organisms less sensitive to such imbalances should be employed. The use of L standards is advisable to eliminate possible activity of the D isomer.

With an isoleucineless strain of *Neurospora crassa*, it has been demonstrated that β-methyl-α-ketovaleric acid, the keto derivative of isoleucine, inhibits the conversion of the keto acid analogue of valine to valine (19). This may be due to saturation of the surface of the enzyme which reductively aminates this keto acid to valine by the structurally related isoleucine derivative. A similar mechanism may account for the relationships described here. Isoleucine may be utilized as a peptide or a similar derivative and its incorporation into such an active intermediate may be mediated by an enzyme which is effectively blocked by the homologous or isomeric amino acids.

Lyman *et al.* (20) have recently reported that the D isomer of isoleucine is utilized by *Lactobacillus arabinosus* when vitamin B_6 is present in the form of pyridoxamine; utilization was greater when leucine was present at 0.4 mg. than at 2.0 mg. per 10 ml. tube. The concentration of leucine used in our studies (11) is one-half the level reported to inhibit utilization of D-isoleucine. The low levels used in these studies are comparable to those found by Lyman *et al.* (20) to enhance utilization of the D form. The anomalous results obtained in assaying samples, with a medium containing high concentrations of valine and leucine and in the presence of pyridoxal, when DL-isoleucine was used for the standard curve, can largely be explained by a combination of the antagonism described here and the variable availability of D-isoleucine for *Lactobacillus arabinosus* shown by Lyman *et al.* (20).

When aspartic acid-glutamic acid ratios were calculated for the data of Hac *et al.* (12), inhibition indices ranged from 30 to 200, depending on pH, weight of inoculum, length of incubation, and whether ammonium salts were present. The inhibition indices found here essentially confirm their work.

Waelsch *et al.* (13), using a 20 hour turbidimetric assay, found that at a concentration of 0.82×10^{-3} M glutamic acid, 24×10^{-3} M oxalacetate at pH 5.7 or 9.5×10^{-3} M $NaHCO_3$ at pH 7.4 would inhibit completely the growth of *Lactobacillus arabinosus*. The inhibition due to oxalacetate could be reversed by a 4-fold increase in concentration of glutamic acid or by 7.0×10^{-5} M glutamine while $NaHCO_3$ inhibition was reversed by a 3-fold increase of glutamic acid or by 2.7×10^{-6} M glutamine. They attributed the inhibition to carbon dioxide, which appeared to prevent the amidation of glutamic acid to glutamine. The competitive aspartic acid-glutamic acid growth inhibition obtained with *L. arabinosus* appears to be a function of the enzyme system which converts glutamic acid to glutamine. Aspartic acid or asparagine may inhibit this reaction by competing with glutamic acid for the enzyme catalyzing this conversion. In our studies, glutamine reversed aspartic acid inhibition, but failed to overcome bicarbonate inhibition. From these results, it appears that these inhibitors do not act by a common mechanism. Aspartic acid is a more effective inhibitor than sodium bicarbonate. Interpretation of the data of Waelsch *et al.* (13) is complicated by the presence of aspartic acid in the medium. Calculation of the inhibition indices showed that asparagine, methionine sulfoxide (21), $NaHCO_3$ (13), oxalacetate (13), and aspartic acid were 200 to 400, 75, 30, 30, and 20, respectively. In these studies, the index for sodium bicarbonate was approximately 50.

By replacing the aspartic acid in the uniform medium (11) for amino acid assays by one-half as much L- or DL-asparagine, a dose-response curve to glutamic acid is obtained which is much more nearly linear and whose slope is largely independent of the size of the inoculum. Such a modified medium has proved highly satisfactory for the determination of glutamic acid.

SUMMARY

1. The metabolism of *Lactobacillus arabinosus* is affected by the balance of concentrations of leucine, isoleucine, valine, and methionine present in the medium.

2. When isoleucine was the limiting amino acid, high concentrations of leucine, valine, and methionine in decreasing order of effectiveness caused inhibition of growth. Alanine, threonine, and serine did not cause inhibition when added at high levels, indicating reaction specificity.

3. When leucine was limiting, isoleucine inhibited growth more than valine and when valine was limiting, isoleucine inhibited growth more than leucine.

4. The growth of *Leuconostoc mesenteroides* P-60 was also affected by imbalances of these amino acids. However, the concentrations necessary to inhibit this organism were approximately 5 times greater than those required to inhibit *Lactobacillus arabinosus*.

5. The growth of *Lactobacillus arabinosus*, with glutamic acid limiting, was inhibited by aspartic acid or asparagine; the former was the more effective antagonist. It appears that these amino acids inhibit by preventing the small amounts of glutamic acid present from being amidated to glutamine. This organism is much less sensitive to this inhibition below pH 7.

6. The uniform medium of Henderson and Snell (11) should be modified for the glutamic acid assay when *Lactobacillus arabinosus* is used by replacing the aspartic acid with asparagine at a lower concentration.

BIBLIOGRAPHY

1. Woods, D. D., *Brit. J. Exp. Path.*, **21**, 74 (1940).
2. Fildes, P., *Lancet*, **1**, 955 (1940).
3. Shive, W., and Macow, J., *J. Biol. Chem.*, **162**, 451 (1946).
4. Gordon, J., and M'leod, J. W., *J. Path. and Bact.*, **29**, 13 (1926).
5. Nielsen, N., and Hartelius, V., *Biochem. Z.*, **295**, 211 (1938).
6. Pollack, M. A., and Lindner, M., *J. Biol. Chem.*, **143**, 655 (1942).
7. Hutchings, B. L., and Peterson, W. H., *Proc. Soc. Exp. Biol. and Med.*, **52**, 36 (1943).
8. Gladstone, G. P., *Brit. J. Exp. Path.*, **20**, 189 (1939).
9. Meinke, W. W., and Holland, B. R., *J. Biol. Chem.*, **173**, 535 (1948).
10. Feeney, R. E., and Strong, F. M., *J. Am. Chem. Soc.*, **64**, 881 (1942).
11. Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172**, 15 (1948).
12. Hac, L. R., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **159**, 273 (1945).
13. Waelsch, H., Prescott, B. A., and Borek, E., *J. Biol. Chem.*, **172**, 343 (1948). Borek, E., and Waelsch, H., *Federation Proc.*, **7**, 147 (1948).
14. Lyman, C. M., Kuiken, K. A., Blotter, L., and Hale, F., *J. Biol. Chem.*, **157**, 395 (1945).
15. Baumgarten, W., Mather, A. N., and Stone, L., *Cereal Chem.*, **22**, 514 (1945).
16. Henderson, L. M., Brickson, W. L., and Snell, E. E., *J. Biol. Chem.*, **172**, 31 (1948).
17. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, **166**, 417 (1946).
18. Smith, E. L., and Greene, R. D., *J. Biol. Chem.*, **172**, 111 (1948).
19. Bonner, D., *J. Biol. Chem.*, **166**, 545 (1946).
20. Lyman, C. A., and Kuiken, K. A., *Federation Proc.*, **7**, 170 (1948).
21. Borek, E., Miller, H. K., Sheiness, P., and Waelsch, H., *J. Biol. Chem.*, **163**, 347 (1946).

REACTION OF *peri*-NAPHTHINDAN-2,3,4-TRIONE HYDRATE WITH *l*-ASCORBIC ACID AND OTHER ENEDIOL COMPOUNDS, WITH A NOTE ON THE ISOLATION OF DEHYDRO-*l*-ASCORBIC ACID

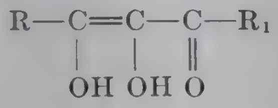
BY RADWAN MOUBASHER

(*From the Faculty of Science, Fouad Ist University, Cairo, Egypt*)

(Received for publication, January 23, 1948)

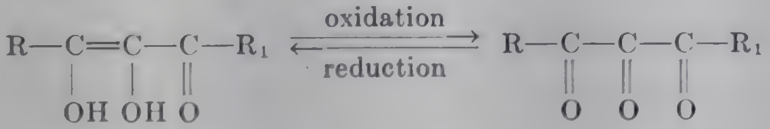
When solutions of *peri*-naphthindan-2,3,4-trione hydrate (1) (II) and ascorbic acid (I), reductone, or dihydroxymaleic acid are mixed at a constant temperature of 45° for about 6 hours, or heated on a boiling water bath for 3 minutes and then cooled, definite silky red crystalline precipitates are formed. These precipitates, which proved to be dihydroxy-*peri*-naphthindenone (III), are similar to the compound prepared by Errera (2), using the action of hydrogen sulfide on *peri*-naphthindan-2,3,4-trione hydrate, and have the same melting point; the mixed melting point was not depressed on addition of an authentic specimen. The same characteristic deep blue coloration is also obtained when the substance is treated with sodium hydroxide solution.

l-Ascorbic acid, reductone, and dihydroxymaleic acid belong, according to their structures and properties, to the class of "reductones," which are characterized by the following constitution:



$$\mathrm{R-\underset{\displaystyle OH}{\underset{|}{C}}=\underset{\displaystyle OH}{\underset{|}{C}}-\underset{\displaystyle O}{\underset{\|}{C}}-R_1}$$

The formulae of these compounds contain a common grouping with that of reductone, which explains their similar behavior, and all compounds of this class form reversible oxidation-reduction systems.

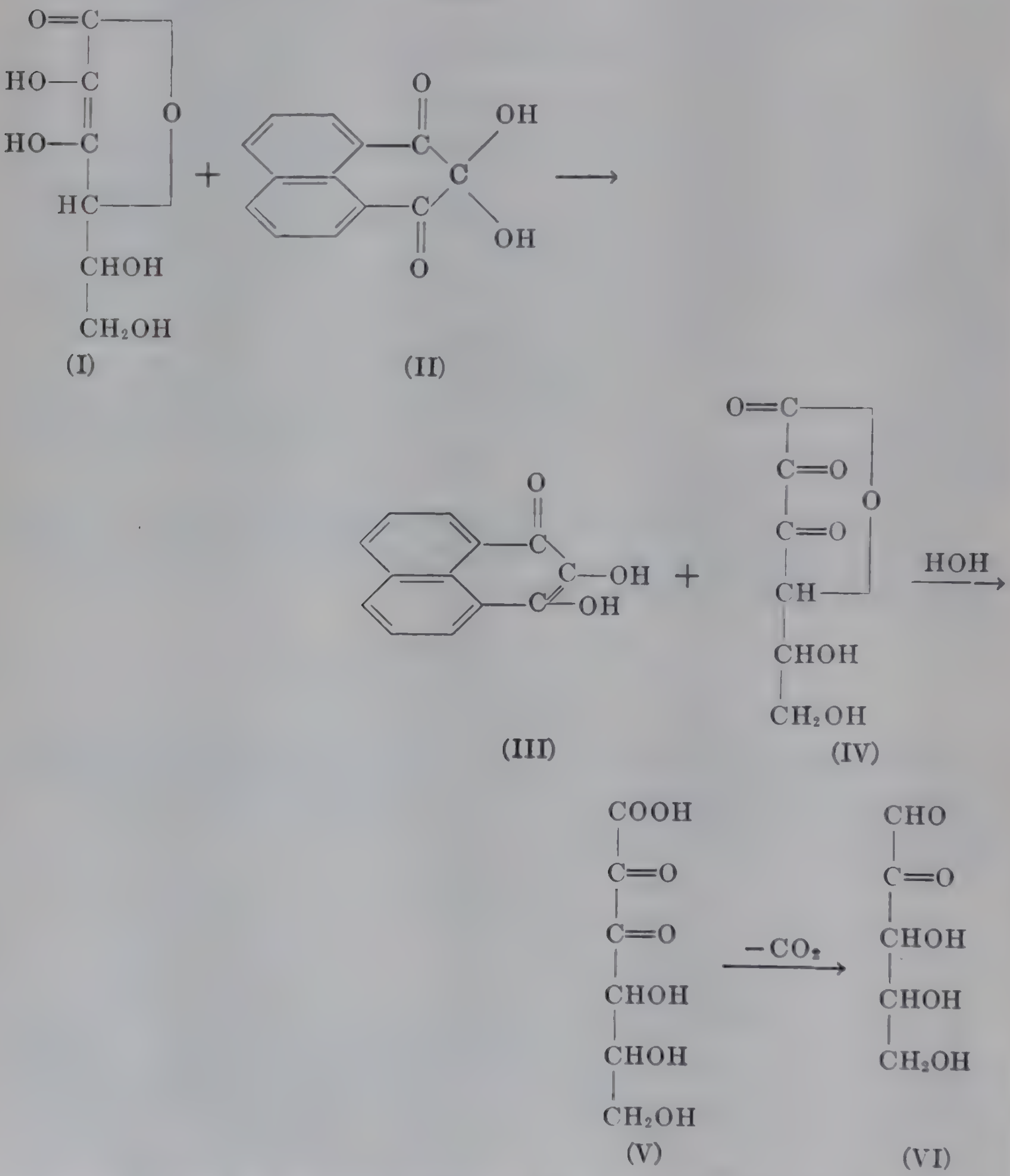


$$\mathrm{R-\underset{\displaystyle OH}{\underset{|}{C}}=\underset{\displaystyle OH}{\underset{|}{C}}-\underset{\displaystyle O}{\underset{\|}{C}}-R_1} \xrightleftharpoons[\text{reduction}]{\text{oxidation}} \mathrm{R-\underset{\displaystyle O}{\underset{\|}{C}}-\underset{\displaystyle O}{\underset{\|}{C}}-\underset{\displaystyle O}{\underset{\|}{C}}-R_1}$$

It follows from these facts that the oxidation of *l*-ascorbic acid by *peri*-naphthindan-2,3,4-trione hydrate stops at the stage of dehydro-*l*-ascorbic acid (IV), since a mixture of dehydro-*l*-ascorbic acid and *peri*-naphthindan-2,3,4-trione hydrate gives no precipitate of dihydroxy-*peri*-naphthindenone. During this reaction and the period of heating at about 45°, there is evolution of carbon dioxide. This is due to decarboxylation of the dehydro-*l*-ascorbic acid after hydrolysis of the lactone bridge, forming *l*-

xylo-2,3-diketohexonic acid (V) as an intermediate product and finally *l*-xylosone (VI) (*cf.* West and Rinehart (3), Koppanyi, Vivino, and Veitch (4)).

In the case of reductone, the stage of oxidation stops at the formation of mesoxalaldehyde, which is identified in the form of its diphenylhydrazone. Dihydroxymaleic acid is oxidized to diketotartaric acid, which is identified in the form of its osazone.



The chemistry of dehydro-*l*-ascorbic acid, the primary reversible oxidation product of vitamin C, has hitherto been mainly investigated by utilizing solutions of the freshly oxidized vitamin. The labile nature of this substance has, however, prevented its isolation hitherto in a pure state, although Karrer, Salomon, and Schöpp (5) and Crook and Morgan (6) have prepared it in a pure but amorphous form. Kenyon and Munro (7) found recently that when the solid product obtained by the method of Hirst and Woodward is intensively dried and dissolved in ethanol at room temperature the solution on standing deposits a crystalline colorless compound which has the characteristics of anhydrous dehydro-*l*-ascorbic acid.

During the action of *peri*-naphthindan-2,3,4-trione hydrate (II) on freshly crystallized *l*-ascorbic acid (I) in aqueous solutions at 20° for 1 hour, it has been found that after separation of dihydroxy-*peri*-naphthindenone (III) and concentration of the filtrate to dryness, followed by treatment with ethanol at 0°, anhydrous dehydro-*l*-ascorbic acid (IV) is isolated in a crystalline state.

It has also been found *in vitro* that dehydro-*l*-ascorbic acid reacts on α-amino acids at the boiling point of water with the formation of ammonia, carbon dioxide, and the corresponding aldehydes with 1 carbon atom less (*cf.* von Euler, Karrer, and Zehender (8), Abderhalden (9)). This degradation reaction has been carried out under physiological conditions of temperature and pH. According to this evidence, it appears reasonable to believe that vitamin C deficiencies which occur quite often may be due to intestinal destruction of the vitamin, partially by hydrolysis followed by decarboxylation of the dehydro-*l*-ascorbic acid and partially to its action on the α-amino acids. This will explain among other reasons the relatively high requirements of this vitamin on the weight basis compared to the daily needs of man and animals for the other vitamins.

Reaction of peri-Naphthindan-2,3,4-trione Hydrate with l-Ascorbic Acid—0.5 gm. of ascorbic acid mixed with 0.6 gm. of pulverized *peri*-naphthindan-2,3,4-trione hydrate in 25 cc. of water is heated in a boiling water bath for 3 minutes and quickly cooled; a red silky crystalline precipitate is obtained, which is filtered off, washed several times with cold water, and then dried; m.p. 258°; yield 0.42 gm. This substance proved to be dihydroxy-*peri*-naphthindenone by mixed melting point with an authentic specimen prepared according to Errera (1, 2) and also by the characteristic intense blue color which is obtained on treatment with sodium hydroxide solution.

$C_{13}H_8O_3$. Calculated, C 73.5, H 3.7; found, C 73.6, H 3.8

Reaction of peri-Naphthindan-2,3,4-trione Hydrate with l-Ascorbic Acid at 45°—0.5 gm. of *l*-ascorbic acid is dissolved in 10 cc. of water and mixed with 0.6 gm. of pulverized *peri*-naphthindan-2,3,4-trione hydrate dissolved in 15 cc. of water. The vessel containing the mixture is arranged so that a continual current of pure hydrogen is passed through it, and it is placed on a constant bath of 40° for 10 hours. It is connected to a bubbler containing barium hydroxide to provide for titration of the amount of carbon dioxide. From 0.5 gm. of ascorbic acid treated with 0.6 gm. of *peri*-naphthindan-2,3,4-trione hydrate at 45°, 0.5 gm. of carbon dioxide was evolved in 6 hours. The solid dihydroxy-*peri*-naphthindenone is separated by filtration and the clear filtrate is concentrated *in vacuo* to a syrupy liquid. This is treated with a phenylhydrazine mixture (0.4 gm. of phenylhydrazine hydrochloride mixed with 0.6 gm. of sodium acetate and 4 cc. of distilled water). *l*-Xylosazone has been obtained in delicate yellow needles and melts at 164°, with decomposition. This is proved by the melting point and mixed melting point of the osazone.

Reaction of peri-Naphthindan-2,3,4-trione Hydrate with Dehydro-l-ascorbic Acid—0.5 gm. of ascorbic acid dissolved in 10 cc. of distilled water is treated with iodine-potassium iodide solution (1.4 gm. of iodine in 2.5 gm. of potassium iodide dissolved in 10 cc. of water) until it is transformed into dehydro-*l*-ascorbic acid. The hydrogen iodide is removed by the addition of silver carbonate and filtration. 0.6 gm. of pulverized *peri*-naphthindan-2,3,4-trione hydrate is added with 25 cc. of water and heated in a boiling water bath for 3 minutes and then cooled; no reaction takes place. The whole solution is concentrated *in vacuo* and then allowed to cool, when a yellowish crystalline precipitate is obtained, which proved to be *peri*-naphthindan-2,3,4-trione hydrate by melting point, 273° with decomposition, and mixed melting point determinations and by color tests with sodium hydroxide solution. The same experiment has been repeated with the same amounts in the presence of a current of pure hydrogen. The reaction mass was placed at 45° for about 6 hours and the amount of CO_2 has been calculated as above and found to correspond to 0.4 gm.

Action of peri-Naphthindan-2,3,4-trione Hydrate with Reductone—0.3 gm. of reductone (*cf.* von Euler and Martius) (10)) with 0.6 gm. of pulverized *peri*-naphthindan-2,3,4-trione hydrate in 25 cc. of distilled water is heated over a flame for 3 minutes and cooled; a red silky crystalline precipitate is obtained which is filtered off and recrystallized from ethyl alcohol; the melting point, 258°, and mixed melting point determinations with an authentic specimen proved the compound to be dihydroxy-*peri*-naphthindenone. Yield, 0.35 gm. The filtrate is concentrated *in vacuo* and treated with the phenylhydrazine mixture. The red substance which separates is recrystallized from methyl alcohol to give a red crystalline substance melting at 176°; the substance proved to be the diphenylhydrazone of mesoxalaldehyde by melting point and mixed melting point determinations with material prepared according to Pechmann (11).

Action of peri-Naphthindan-2,3,4-trione Hydrate with Dihydroxymaleic Acid—0.4 gm. of dihydroxymaleic acid (12) is mixed with 0.6 gm. of pulverized *peri*-naphthindan-2,3,4-trione hydrate in 25 cc. of distilled water and heated over a water bath at 45° for some hours and then cooled. A red, silky, crystalline precipitate obtained was filtered off and recrystallized from ethyl alcohol, forming red needles of melting point 258° which was not depressed when the substance was mixed with dihydroxy-*peri*-naphthindenone. Yield, 0.2 gm. The mother liquor of the reaction mass after separation of the red substance was concentrated *in vacuo* to a syrupy liquid which was chilled in ice for 24 hours. A colorless crystalline substance was obtained, melting at 114°; the mixed melting point was not depressed on admixture with diketotartaric acid. When this was treated with the phenylhydrazine mixture, orange crystals contaminated with impurities were obtained. By fractional crystallization uniform orange needles were separated, m.p. 200°. This osazone has been identified as the phenylhydrazone of diketotartaric acid by melting point and mixed melting point determinations (*cf.* Ziegler and Lacher (13)). The yield was very poor. This is due to the partial decomposition of dihydroxymaleic acid at 50–60°, with liberation of carbon dioxide and the formation of glycolaldehyde (*cf.* Fenton (14)).

Isolation of Dehydro-l-ascorbic Acid (*IV*)—3 gm. of pure *l*-ascorbic acid (I) dissolved in 25 cc. of distilled water were shaken with 3.5 gm. of *peri*-naphthindan-2,3,4-trione hydrate (II) in 150 cc. of distilled water at 20° for 1 hour. A reddish color first formed, followed by precipitation of red, silky, crystalline needles of dihydroxy-*peri*-naphthindenone (III). The whole mass was cooled at 0° and filtered off. When the filtrate was concentrated at 30° and 4 mm., a viscous yellowish syrup was obtained; this was dried *in vacuo* at 50° for 20 minutes. The resultant glassy substance was then left under a vacuum for some hours until of constant weight. This solid mass was powdered and shaken with 10 cc. of absolute alcohol and kept at 0° for 24 hours; anhydrous dehydro-*l*-ascorbic acid (IV) was obtained in fine colorless crystalline needles, m.p. 220° with decomposition, which were identified by mixed melting point determinations with an authentic sample prepared according to Kenyon and Munro (7). Yield, 0.8 gm.

Action of Dehydro-l-ascorbic Acid on Phenylaminoacetic Acid under Physiological Conditions of Temperature and pH—Dehydroascorbic acid (0.5 gm.), prepared by the method mentioned above, and phenylaminoacetic acid (0.3 gm.) are placed with water (75 cc.) in a flask fixed in a thermostat adjusted at 37°. The flask is provided with a delivery tube dipping into a solution of phenylhydrazine hydrochloride (0.4 gm. in 30 cc. of alcohol). After 72 hours, the contents of the reaction flask which had a strong odor of benzaldehyde are subjected to distillation under reduced pressure, carbon dioxide being bubbled through. The distillation is controlled in such a way that a thermometer dipping into the reaction mixture does not rise over 37°; the receiver is cooled in an ice-salt mixture and connected to a trap containing the phenylhydrazine mixture mentioned above to retain any benzaldehyde which might have escaped. The distillate (about 50 cc.) is treated with the same phenylhydrazine mixture. Benzaldehyde phenylhydrazone was obtained which melted at 158° and showed no depression in the mixed melting point when mixed with an authentic specimen. Yield, 0.1 gm.

SUMMARY

1. Ascorbic acid, reductone, and dihydroxymaleic acid react with *peri*-naphthindan-2,3,4-trione hydrate at 45° for a long time, giving dihydroxy-*peri*-naphthindenone.
2. Ascorbic acid is oxidized to dehydroascorbic acid, followed by hydrolysis and decarboxylation with the formation of *l*-xylosone.
3. Reductone and dihydroxymaleic acid give respectively mesoxalaldehyde and diketotartaric acid, identified by their phenylhydrazone derivatives.
4. Dehydroascorbic acid has been isolated in a pure crystalline state.
5. Dehydroascorbic acid has been found to undergo Strecker degradation under physiological conditions of temperature and pH.

BIBLIOGRAPHY

1. Errera, G., *Gazz. chim. ital.*, **43**, 583 (1913).
2. Errera, G., *Gazz. chim. ital.*, **43**, 288 (1913).
3. West, E. S., and Rinehart, R. E., *J. Biol. Chem.*, **146**, 105 (1942).
4. Koppanyi, T., Vivino, A. E., and Veitch, F. P., Jr., *Science*, **101**, 541 (1945).
5. Karrer, P., Salomon, H., and Schöpp, K., *Nature*, **131**, 800 (1933).
6. Crook, E. M., and Morgan, E. J., *Biochem. J.*, **38**, 10 (1944).
7. Kenyon, J., and Munro, N., *J. Chem. Soc.*, 158 (1948).
8. von Euler, H., Karrer, P., and Zehender, F., *Helv. chim. acta*, **17**, 157 (1934).
9. Abderhalden, E., *Fermentforschung*, **15**, 522 (1938).
10. von Euler, H., and Martius, C., *Ann. Chem.*, **505**, 73 (1933).
11. Pechmann, H., and Jenisch, K., *Ber. chem. Ges.*, **24**, 3255 (1891).
12. Fenton, H. J. H., *J. Chem. Soc.*, **65**, 899 (1894).
13. Ziegler, J. H., and Lacher, M., *Ber. chem. Ges.*, **20**, 836 (1887).
14. Fenton, H. J. H., *J. Chem. Soc.*, **87**, 817 (1905).

THE METABOLISM OF 4-DIMETHYLAMINOAZOBENZENE BY RAT LIVER HOMOGENATES*

BY G. C. MUELLER† AND J. A. MILLER

(From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison)

(Received for publication, July 16, 1948)

The intact rat metabolizes the hepatic carcinogen 4-dimethylaminoazobenzene in several ways. Small amounts of the ingested dye and two demethylated derivatives, 4-monomethylaminoazobenzene and 4-aminoazobenzene, have been found in the liver and excreta (1). The latter two dyes and an unidentified aminoazo dye also occur in the liver firmly combined with protein; these bound dyes appear to be intimately associated with the carcinogenic process induced by the parent dye (2). Other tissues contain only 4-aminoazobenzene; this is also the only dye detectable in the blood and it is found entirely in the sedimented blood cells (1). In the urine approximately 50 per cent of the ingested dye can be accounted for in the form of two conjugated amines, *p*-phenylenediamine and *p*-aminophenol; small amounts of several other monophenylamines and demethylated hydroxyazo dyes are also present (3, 4). Thus 4-dimethylaminoazobenzene is subject to at least three metabolic reactions in the rat: demethylation of the dimethylamino group, hydroxylation, principally at the 4′ position, and reductive cleavage of the azo linkage. The exact sequence of these reactions and the extent to which each occurs are unknown.

The only previously published work on the metabolism of 4-dimethylaminoazobenzene by tissue *in vitro* demonstrated that this dye is destroyed by surviving rat liver slices (5); no metabolites of the dye were found in these reactions. The present communication deals with the metabolism of this carcinogen in fortified rat liver homogenates. In these reaction mixtures it has been possible to demonstrate that stepwise demethylation of the dye also occurs *in vitro* and that a new metabolite, 4′-hydroxy-4-dimethylaminoazobenzene, is formed by hydroxylation of the parent dye. The participation of several cell constituents, particularly diphosphopyridine nucleotide (DPN), in these reactions has also been studied.

Methods

Young adult Sprague-Dawley rats maintained on a grain diet were killed by decapitation and liver samples transferred immediately to ice-

---

* This work was supported by grants from the National Cancer Institute and the Jane Coffin Childs Fund for Medical Research.

† Finney-Howell Fellow, 1947–48.

cold isotonic KCl. In later experiments it was found that higher activity and substrate response were obtained with a preliminary fasting period of 18 to 24 hours. 10 per cent homogenates were prepared in isotonic KCl containing 8.0 ml. of 0.02 M K_2CO_3 per liter (6). All reactants were maintained at 0° prior to incubation.

A typical reaction mixture contained the following ingredients, each adjusted to pH 7.4 in a final volume of 3 ml.: 0.2 to 0.4 ml. of 10 per cent homogenate, 0.4 ml. of 0.03 M hexose diphosphate,[1] 0.1 ml. of 1 per cent diphosphopyridine nucleotide[1] (65 per cent assay), 0.2 ml. of 0.6 M nicotinamide, 0.1 ml. of 0.1 M $MgCl_2$, 0.4 ml. of 0.5 M KCl, and 0.5 ml. of 0.1 M K_2HPO_4-KH_2PO_4 buffer at pH 7.4. The 4-dimethylaminoazobenzene (20 to 50 γ) was customarily added last in 0.1 ml. of aldehyde-free 95 per cent ethanol. The most accurate addition and best dispersion of the dye were obtained by discharging the dye solution from a micro blow pipette. The mixtures were incubated in open 25 ml. Erlenmeyer flasks with mechanical shaking in a water bath held at 37.5°.

Estimation of Over-All Destruction of Dye—A rapid estimation of the residual dyes in the reaction mixtures was made by adding 3 ml. of a 20 per cent solution of trichloroacetic acid in 1:1 acetone-ethanol. This reagent precipitated the protein, extracted the adsorbed dyes, and developed the characteristic acid color (7, 8) of the dyes. The dye solutions were diluted to the proper optical density with equal parts of the trichloroacetic acid reagent and water. After light centrifugation the optical density of the solutions was determined at 520 mμ in a Cenco-Sheard spectrophotelometer adapted to the use of matched 13 mm. × 100 mm. Pyrex culture tubes. The solutions obeyed Beer's law up to at least 2.9 γ per ml. The tissue blanks, which were of low optical density, were deducted and the residual dyes expressed as equivalents of 4-dimethylaminoazobenzene. This rapid method gave reproducible results at the low tissue concentrations used (20 to 50 mg. per 3.0 ml. of reaction mixture).

Fractionation and Estimation of Possible Azo Metabolites of 4-Dimethylaminoazobenzene—Methods were devised by which the basic dyes, 4-dimethylaminoazobenzene, 4-monomethylaminoazobenzene, 4-aminoazobenzene, and the 4′-hydroxy derivatives of these dyes could be separately determined in the reaction mixtures. Recrystallized and chromatographically pure samples of the dyes prepared in this laboratory (9) were used. For fractionation of these possible metabolites the volume of the reaction mixture was doubled and incubated in open 50 ml. Erlenmeyer flasks. The reactions were stopped by the addition of approximately 0.5 gm. of solid $BaCl_2$ and 6.0 ml. of acetone. After standing for 20 to 40 minutes to complete the extraction of the dyes from the precipitate, 8.0 ml. of benzene were added and the mixture shaken vigorously for $\frac{1}{2}$ to 1 minute. The basic and hydroxy dyes were thus extracted quantitatively into the acetone-benzene phase. Any emulsions that formed were broken by centrifugation in stoppered tubes and a 5 ml. aliquot of the upper phase was taken for analysis. This aliquot represented 41 per cent of the dyes in the reaction mixture. For uniformity of expression, the results are reported as the dye present in 3.0 ml. of reaction media.

---

[1] The hexose diphosphate, DPN, and ATP were generously supplied by Dr. G. A. LePage.

The basic dyes were measured in the extract by removing the solvent *in vacuo* and redissolving the residue in Skellysolve B.[2] The dyes were separated from one another by chromatographing this solution on a 4 mm. × 100 mm. column of activated alumina. Each dye was separately eluted and determined by its color in strong acid as previously described (7).

Since the hydroxy dyes were adsorbed on the top of the alumina column and could not be quantitatively eluted, a separate 5 ml. aliquot of the initial extract was used for the estimation of these dyes. The solvent was removed *in vacuo* and the residue taken up in 5 ml. of Skellysolve B; this addition was followed by 5 to 10 ml. of 7 N HCl. The mixture was stirred mechanically for 1 minute to transfer all of the azo dyes to the acidic phase. The supernatant layer of Skellysolve B containing interfering tissue material was then decanted and a second extraction with this solvent performed. The residual solvent was removed under reduced pressure and the contents neutralized to approximately pH 3.0 and buffered by the addition of 1 ml. of 3 M KH_2PO_4 at pH 3.0. Exactly 6.0 ml. of benzene were added and the contents shaken for $\frac{1}{2}$ to 1 minute or until no acid color was visible in the aqueous phase. A 5 ml. aliquot of the extract, representing 34 per cent of the hydroxy dyes, was taken to dryness, redissolved in 0.1 to 0.2 ml. of benzene, and diluted with 2 ml. of Skellysolve B; this solution was resolved by chromatographic adsorption.

Hyflo Super-Cel (Johns-Manville) which had been washed with methanol and dried at 37° for 12 to 15 hours proved to be an excellent adsorbent for the chromatographic separation of the hydroxylated dyes. The basic dyes present in the extracts were readily eluted with Skellysolve B, and the hydroxy dyes formed slowly moving zones which were eluted successively with Skellysolve B containing 0.3 per cent of isopropanol. The dyes elute in an order similar to that of the basic dyes from alumina: 4′-hydroxy-4-dimethylaminoazobenzene > 4′-hydroxy-4-monomethylaminoazobenzene > 4′-hydroxy-4-aminoazobenzene. The N-methyl hydroxy dyes were extracted into 2 N HCl and yielded purple solutions with absorption maxima at 545 mμ and 535 mμ respectively for the dimethyl and monomethyl compounds. The 4′-hydroxy-4-aminoazobenzene was extracted into 7 N HCl and yielded a yellow solution with an absorption maximum at 465 mμ. Experiments with 1 γ of either methylated dye proved that recoveries of 90 to 93 per cent from tissue could be consistently achieved. Due to the higher water solubility of 4′-hydroxy-4-aminoazobenzene, the recoveries of 2 γ quantities of this dye averaged 70 per cent. All of these dyes obeyed Beer's law in acid solution.

---

[2] A commercial petroleum ether, b.p. 66–68°.

Attempts to use adsorption on Super-Cel to effect an initial separation of the hydroxy dyes from the basic dyes failed, since poor recoveries of the two basic dyes, 4-monomethylaminoazobenzene and 4-aminoazobenzene, occurred during the extraction of the dyes from acid solution at pH 3 with benzene. These conditions were required for the efficient extraction of the amphoteric hydroxyaminoazo dyes.

TABLE I

Effect of Fumarate and Tissue Extract on Destruction of 4-Dimethylaminoazobenzene by Rat Liver Homogenates

200 mg. of rat liver homogenate (unfasted rat), 0.5 ml. of 0.1 M K_2HPO_4, pH 7.4, 0.5 ml. of 0.0125 M fumarate, 1.0 ml. of rat muscle extract, 14.8 γ of dye in 0.1 ml. of ethanol, and water to a final volume of 3.0 ml. Incubated for 30 minutes.

Factors added			Over-all destruction of dye
Homogenate	Muscle extract	Fumarate	
			per cent
+	−	−	39
+	+	−	54
+	−	+	63
+	+	+	89
−	+	+	3

Results

In preliminary experiments only slight or no destruction of 4-dimethylaminoazobenzene was obtained even with 400 mg. of unfortified liver homogenate. By observing cold temperature precautions, the activity of the homogenate was improved. The addition of 1.0 ml. of 4:1 muscle extract (10) and 0.002 M fumarate enables much less homogenate to destroy a considerable quantity of dye (Table I). The requirements of the system for optimum ability to destroy dye were then studied.

The data in Table II demonstrate that DPN is essential for the destruction of dye, although the rate of destruction falls off rapidly with the coenzyme alone. The addition of 0.2 ml. of 0.6 M nicotinamide optimally decreased or eliminated the requirement for added DPN. However, as a precaution against variation in the preservation of the DPN in the homogenates, 1.0 mg. of DPN assaying 65 per cent, or its equivalent, was always added. No destruction of azo dye could be demonstrated in three experiments when reduced DPN was employed in the absence of liver homogenate. For example, 790 γ of DPN (90 per cent reduced), prepared according to Green and Dewan (11), failed to react with 21 γ of 4-dimethylaminoazobenzene when incubated with or without nicotinamide, $MgCl_2$, and isotonic KCl buffered at pH 7.4 with KH_2PO_4.

TABLE II

Effect of DPN, Nicotinamide, and ATP on Destruction of 4-Dimethylaminoazobenzene by Rat Liver Homogenates

Systems as under "Methods" with 26.3 γ of dye and 0.4 ml. of homogenate (unfasted rat) per 3.0 ml. of reaction mixture incubated for 20 minutes. The molarity of the factor is the final concentration in the reaction mixture.

Factors added			Over-all destruction of dye
DPN, 0.00023 M	Nicotinamide, 0.04 M	ATP, 0.0003 M	
			per cent
−	−	−	4
+	−	−	35
+	+	−	63
+	+	+	60
+	−	+	37
−	−	+	13
−	+	−	58
−	+	+	57

TABLE III

Effect of Oxidizable Substrates on Destruction of 4-Dimethylaminoazobenzene by Rat Liver Homogenates

Systems as under "Methods" with 45.5 γ of dye and 0.3 ml. of homogenate (fasted rat) incubated for 20 minutes. The molarity is the final concentration in the reaction mixture.

Substrate added (0.004 M)	Over-all destruction of dye
	per cent
None	5
Hexose diphosphate	47
Succinate	30
Malate	34

A greater destruction of dye occurred when an oxidizable substrate was added. Malate, fumarate, succinate, α-ketoglutarate, and hexose diphosphate, each at a final concentration of 0.004 M, increased the over-all destruction of dye. In experiments comparing hexose diphosphate, malate, and succinate, the best response was obtained with hexose diphosphate (Table III). Oxalacetate occasionally stimulated the rate of destruction, but its effect was variable. No dye was destroyed when succinate, malate, or hexose diphosphate was added in the absence of DPN and nicotinamide.

Magnesium ions were necessary for maximum activity. A final concentration of 0.003 M $MgCl_2$ increased the dye destruction by 25 per cent.

A requirement for adenosine triphosphate (ATP)[1] could not be demonstrated in the presence of DPN and nicotinamide (Table II), and only a slight stimulation occasionally occurred when ATP was added alone. If high energy phosphate is involved in this system, the needs are apparently supplied by the tissue and added reactants.

TABLE IV

Effect of Various Enzyme Inhibitors on Destruction of 4-Dimethylaminoazobenzene by Rat Liver Homogenates

Systems as under "Methods" with 28.6 γ of dye and 0.4 ml. of homogenate (unfasted rat) incubated for 20 minutes. The molarity of the inhibitor is the final concentration in the reaction mixture. ATP at a final concentration of 0.0003 M was present in the mixtures under Experiment 3.

Experiment No.	Inhibitor added	Concentration	Over-all destruction of dye
		M	*per cent*
1	None		48
	Potassium iodoacetate	0.001	42
	Sodium azide	0.01	29
	p-Aminophenol	0.001	32
	Potassium fluoride	0.01	48
2	None		67
	Potassium malonate	0.01	63
	Hydroxylamine	0.01	43
	Sodium cyanide	0.01	27
3	None		55
	Guanidinoacetic acid	0.003	55
	DL-Methionine	0.003	55
	Potassium benzoate	0.003	53
	Atabrine	0.001	37

Heating the liver homogenates for 1 minute in a boiling water bath completely inactivated the system. The effect of adding various enzyme inhibitors to the system is presented in Table IV. Definite inhibition was produced by iodoacetate, *p*-aminophenol, hydroxylamine, cyanide, azide, and atabrine. Fluoride, semicarbazide, and malonate were without effect at the concentrations tried. The addition of DL-methionine, benzoate, or guanidinoacetic acid in the presence of ATP also did not alter the reaction rate or the final distribution of the metabolites of the dye.

During the reaction appreciable quantities of 4-monomethylaminoazobenzene, 4-aminoazobenzene, and 4′-hydroxy-4-dimethylaminoazobenzene were formed from the added 4-dimethylaminoazobenzene. Generally the levels of the latter two metabolites increased with the length of the incubation period, whereas 4-monomethylaminoazobenzene was frequently present in highest concentration after the first 5 to 10 minutes (Table V). Traces of a dye similar in its adsorption properties on Super-Cel to those of 4′-hydroxy-4-monomethylaminoazobenzene were frequently observed; however, no evidence for the presence of any 4′-hydroxy-4-aminoazobenzene was found.

TABLE V

Distribution of Azo Metabolites of 4-Dimethylaminoazobenzene in Fortified Rat Liver Homogenates Incubated with This Dye

Systems as under "Methods" with 0.4 ml. of homogenate (unfasted rat). The figures are micrograms of dye in 3.0 ml. of the reaction mixture.

Added dye or its metabolite*	DPN and nicotinamide omitted				Nicotinamide omitted				Whole system			
	0 min.	10 min.	20 min.	30 min.	0 min.	10 min.	20 min.	30 min.	0 min.	10 min.	20 min.	30 min.
DAB	27.8	26.8	26.0	26.4	27.8	20.2	19.2	20.1	27.8	12.0	4.1	1.3
MAB		0.6	1.2	1.1		1.1	1.4	1.6		1.3	0.6	0.3
AB			0.2	0.2		0.4	0.4	0.5		0.7	1.1	1.0
4′-HO-DAB						1.2	1.2	1.1		2.4	2.6	2.9

* DAB = 4-dimethylaminoazobenzene, MAB = 4-monomethylaminoazobenzene, and AB = 4-aminoazobenzene.

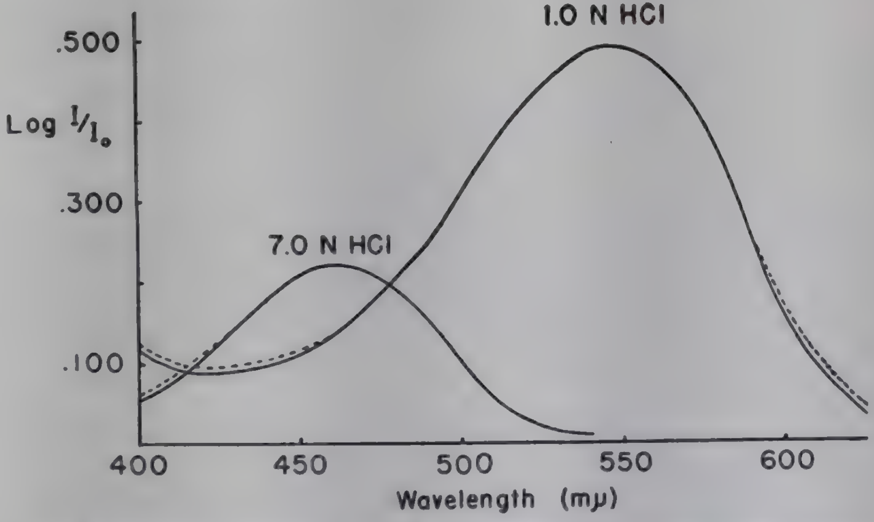


FIG. 1. The absorption spectra of 4′-hydroxy-4-dimethylaminoazobenzene (solid lines) and the acidic metabolite of 4-dimethylaminoazobenzene (broken lines) in 1.0 N and 7.0 N HCl. The concentration of the authentic dye was 7.5 γ and 1.11 γ per ml. respectively. Cell thickness = 1 cm. The curves were drawn so that the densities at 545 mμ (1.0 N HCl) and 465 mμ (7.0 N HCl) coincided.

The identities of the two basic dyes formed from the added dye were established by their absorption spectra in acid and by mixed chromatograms with the known dyes, as previously described (1).

The identity of the acidic dye from the reaction mixture with 4′-hydroxy-4-dimethylaminoazobenzene was established in several ways. First, the absorption spectra of the metabolite in strong and weak acid corresponded closely with the spectra of the authentic compound (Fig. 1). Second, mixed chromatograms of the reaction product with the known dye gave only a single zone with no evidence of resolution. Finally, the metabolite was reductively cleaved with $Na_2S_2O_4$ in acid solution and the amines formed were allowed to react with sodium β-naphthoquinone sulfonate at pH 7 (3). Two Schiff bases were formed, one extractable by benzene and the other subsequently extractable with amyl acetate. These properties and the absorption spectra of the bases (Fig. 2) in these solvents demonstrated that the amines were N,N-dimethyl-*p*-phenylenediamine and *p*-aminophenol, respectively. Approximately equimolar amounts of these amines were produced upon reduction of the acidic dye.

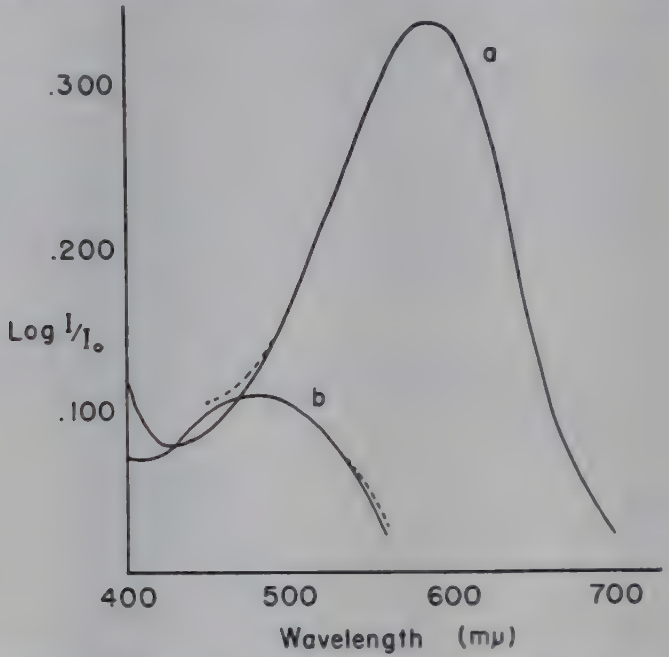


FIG. 2. The absorption spectra of the Schiff bases of the aromatic amines resulting from the reductive cleavage of 4′-hydroxy-4-dimethylaminoazobenzene (solid lines) and the acidic metabolite of 4-dimethylaminoazobenzene (broken lines). Curves *a* = Schiff bases of N,N-dimethyl-*p*-phenylenediamine in benzene; Curves *b* = Schiff bases of *p*-aminophenol in amyl acetate. Cell thickness = 1 cm. The curves were drawn so that the densities at 590 mμ (Curves *a*) and 485 mμ (Curves *b*) coincided.

DISCUSSION

The enzymatic nature of the destruction of 4-dimethylaminoazobenzene by fortified rat liver homogenates is indicated by the observation that the system is inactivated by heat and requires the presence of DPN for activity.

The need of added substrate for maximum activity and the inability of reduced DPN to reduce the dye directly are further evidence that the reactions are catalyzed enzymatically. It is of interest that the livers of rats fed the dye contain less DPN than do livers of normal rats (12), and that the enzymatic destruction of testosterone (13) and α-estradiol (14) by rat liver minces also require DPN.

Presumably the reactions involved in the stepwise demethylation of 4-dimethylaminoazobenzene *in vivo* (1) are identical with those concerned *in vitro*. These may be oxidative demethylations similar to that involved in the enzymatic degradation of N-methylglycine to glycine and formaldehyde by liver mince (15). The stepwise oxidative demethylation of 4-dimethylaminoazobenzene occurs slowly in autoxidizing linoleic acid (16), but it is unknown to what extent a reaction of this type could account for the demethylations occurring in the liver homogenates. If it does occur, it must be catalyzed enzymatically. Transfer of the methyl groups seems unlikely, since the ability to donate methyl groups in the body is possessed by only a few of the naturally occurring methylated substances (17). Furthermore, it has not been possible to alter the carcinogenicity of either 4-dimethylaminoazobenzene or 4-monomethylaminoazobenzene by diets high in methyl donors or acceptors (18–20).

Since riboflavin is a potent inhibitor of the carcinogenic action of 4-dimethylaminoazobenzene (20–22), it is of interest that Kensler (23) recently reported that liver slices from rats fed high levels of this vitamin destroy the dye faster than liver slices from rats deficient in this factor. These facts suggest the possibility that demethylase, a riboflavin enzyme which oxidatively demethylates certain N-methyl-L-amino acids (24), may be involved in the demethylation of the azo dye in the rat liver. Similarly, the riboflavin enzyme quinine oxidase (25) which hydroxylates quinine might be involved in the hydroxylation of the azo dye.

No resolution of the demethylation and hydroxylation reactions in the liver homogenates has been achieved so far. It is apparent that a large fraction of the added dye that disappeared in these reactions was not recovered in the form of the several azo dyes tested for; this decrease may well result from the reductive cleavage of the azo linkages in these dyes. These reductions occur readily *in vivo* (3, 4).

While it is unknown to what extent the enzymatic hydroxylation of 4-dimethyaminoazobenzene in the 4′ position proceeds *in vivo*, this reaction may be of significance, since the hydroxy dye is non-carcinogenic (9). This is in contrast to the stepwise demethylation of the dye in which the first demethylated product is as carcinogenic as the parent dye (18). Thus all of the available evidence still points to an azo dye very closely related to 4-dimethylaminoazobenzene as being the primary carcinogen when the dye is fed (2, 9).

SUMMARY

Methods are described by which the hepatic carcinogen 4-dimethylaminoazobenzene, its demethylated derivatives 4-monomethylaminoazobenzene and 4-aminoazobenzene, and the 4′-hydroxy derivatives of these three dyes can be determined in tissue extracts. With the aid of these methods rat liver homogenates fortified with diphosphopyridine nucleotide, nicotinamide, magnesium ion, and hexose diphosphate have been found to demethylate added 4-dimethylaminoazobenzene and to hydroxylate this dye to form a new metabolite, 4′-hydroxy-4-dimethylaminoazobenzene. More dye disappeared in these reaction mixtures than could be accounted for by the azo metabolites found.

BIBLIOGRAPHY

1. Miller, J. A., Miller, E. C., and Baumann, C. A., *Cancer Res.*, **5**, 162 (1945).
2. Miller, E. C., and Miller, J. A., *Cancer Res.*, **7**, 468 (1947).
3. Miller, J. A., and Miller, E. C., *Cancer Res.*, **7**, 39 (1947).
4. Stevenson, E. S., Dobriner, K., and Rhoads, C. P., *Cancer Res.*, **2**, 160 (1942).
5. Kensler, C. J., Magill, J. W., and Sugiura, K., *Cancer Res.*, **7**, 95 (1947).
6. Potter, V. R., LePage, G. A., and Klug, H. L., *J. Biol. Chem.*, **175**, 619 (1948).
7. Miller, J. A., and Baumann, C. A., *Cancer Res.*, **5**, 157 (1945).
8. Miller, J. A., Sapp, R. W., and Miller, E. C., *J. Am. Chem. Soc.*, in press.
9. Miller, J. A., and Miller, E. C., *J. Exp. Med.*, **87**, 139 (1948).
10. Lardy, H. A., in Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis, 192 (1945).
11. Green, D. E., and Dewan, J. G., *Biochem. J.*, **31**, 1069 (1937).
12. Kensler, C. J., Sugiura, K., and Rhoads, C. P., *Science*, **91**, 623 (1940).
13. Sweat, M. L., and Samuels, L. T., *J. Biol. Chem.*, **173**, 433 (1948).
14. Coppedge, R. L., Segaloff, A., Sarett, H. P., and Altschul, A. M., *J. Biol. Chem.*, **173**, 431 (1948).
15. Handler, P., Bernheim, M. L. C., and Klein, J. R., *J. Biol. Chem.*, **138**, 211 (1941).
16. Rusch, H. P., and Miller, J. A., *Proc. Soc. Exp. Biol. and Med.*, **68**, 140 (1948).
17. du Vigneaud, V., Moyer, A. W., and Chandler, J. P., *J. Biol. Chem.*, **174**, 477 (1948).
18. Miller, E. C., and Baumann, C. A., *Cancer Res.*, **6**, 289 (1946).
19. White, J., and Edwards, J. E., *J. Nat. Cancer Inst.*, **3**, 43 (1942).
20. Kensler, C. J., Sugiura, K., Young, N. F., Halter, C. R., and Rhoads, C. P., *Science*, **93**, 308 (1941).
21. Miller, E. C., Miller, J. A., Kline, B. E., and Rusch, H. P., *J. Exp. Med.*, **88**, 89 (1948).
22. Miller, J. A., *Ann. New York Acad. Sc.*, **49**, 19 (1947).
23. Kensler, C. J., paper presented before the American Association for Cancer Research, Atlantic City, Mar. 13 (1948).
24. Ling, K.-H., and Tung, T.-C., *J. Biol. Chem.*, **174**, 643 (1948).
25. Knox, W. E., *J. Biol. Chem.*, **163**, 699 (1946).

THE ESTIMATION OF MUCIN IN GASTRIC JUICE*

By HERMAN SIPLET, S. A. KOMAROV, and HARRY SHAY

(*From the Samuel S. Fels Research Institute, Temple University School of Medicine, Philadelphia*)

(Received for publication, June 10, 1948)

The importance of mucin as a constituent of gastric mucus and of acid gastric juice has been generally recognized (2). Numerous methods for the quantitative determination of mucin have been described, the estimation of the reducing power having been the method almost universally used. The viscosity, acid-combining power, nitrogen content, and iodine-combining power have each been considered as a measure of mucin concentration; methods based on these properties have been applied to the whole gastric secretion or its contents or to various fractions isolated by ultrafiltration, electrodialysis, and the use of various protein precipitants (3–8). However, none of these methods has gained general acceptance (2).

Mucoitinsulfuric acid is regarded as a characteristic prosthetic group of gastric mucin and many other mucoproteins (9). It has been isolated from hog gastric mucus (10–13) and from pure canine gastric juice (14). Hexuronic acid (12) or, more specifically, glucuronic acid (9, 13) has been established as one of the four components of this polysaccharide. Since (gluc)uronic acid appears to be the most characteristic component of gastric mucin and several reliable methods for its estimation are available, we investigated the possibility of utilizing the estimation of glucuronic acid as a measure of the concentration of mucin in gastric juice. We have found that a modification of Tollens' naphthoresorcinol reaction for glucuronic acid (15) results in a consistent recovery of uronic acid from gastric mucin, mucoitinsulfuric acid, and whole canine gastric juice or mucus.

EXPERIMENTAL

The conclusions and opinions expressed in these studies are based on the results of more than 2800 determinations made in experiments of various types over a period of more than 2 years. The experiments presented here in the form of various tables and figures were selected on the basis of their illustrative value.

Preliminary Experiments

In our preliminary experiments we used the procedure outlined by Maughan, Evelyn, and Browne (16) for the determination of glucuronic acid derivatives in urine as being the least complicated quantitative procedure of the reported modifications of Tollens' naphthoresorcinol reaction. When applied to gastric mucin, mucoitinsulfuric acid, and pure canine gastric juice, it produced a color characterized by the maximal absorption of light transmitted by Rubicon Filter 565. It is well known, however, particularly from the studies of Levene and his associates (9), that rather prolonged hydrolysis with strong acid is required for complete degradation of mucoitinsulfuric acids. Therefore it was necessary to establish conditions of hydrolysis which would result in the optimal recovery of uronic acid from mucin. Boiling for varying lengths of time with approximately 3 N hydrochloric acid in the presence of naphthoresorcinol was used by many investigators for the colorimetric estimation of uronic acids and their various derivatives (17–19). Similarly, boiling with comparable concentrations of hydrochloric acid was found to be successful in effecting hydrolysis of mucoitinsulfuric acid and chondroitinsulfuric acid with the liberation of their monosaccharide components. Accordingly, it was desirable to establish the optimal conditions for the hydrolysis of mucin compatible with the optimal development of color with naphthoresorcinol.

---

* This paper was read at the Fifty-sixth annual meeting of the American Physiological Society in Chicago, May, 1947 (1).

The sources of various preparations employed in this study were as follows:

The glucuronic acid was obtained from the A. D. Mackay Company, New York, and had a warranted purity of less than 5 per cent lactose and a melting point of 146° (uncorrected).

The menthylglucuronic acid was isolated from the urine of menthol-fed rabbits by the method of Williams (20). After the material had been purified by crystallizing it thrice, the melting point was 92.1° (uncorrected).

The gastric mucin was a preparation previously described by one of us (14). It was isolated from 15.4 liters of pure gastric juice obtained from dogs equipped with a gastric fistula and esophagotomy. The ash content was 0.50 per cent, and the elementary composition calculated for the ash-free substance was C 52.68 per cent, H 7.00 per cent, N (Dumas) 14.02 per cent, S (in the form of ethereal sulfate) 0.372 per cent, and P 0.00 per cent. The reducing power (Hagedorn-Jensen method after hydrolysis with N HCl for 6 hours at 100° in a sealed tube) was equivalent to 14.67 per cent glucose. The barium salt of mucoitinsulfuric acid was isolated in a high state of purity in a yield of 4.32 per cent. In all the experiments the preparation of gastric mucin mentioned above was dissolved in 0.02 N sodium hydroxide.

The mucoitinsulfuric acid (acid sodium salt) was isolated from Wilson's "gastric mucin" by the method of Levene and López-Suárez (11) and had an ash content of 4.97 per cent. The elementary composition (calculated for the ash-free substance) was C 41.23 per cent, H 6.20 per cent, N (Dumas) 5.31 per cent, S (in the form of ethereal sulfate) 1.65 per cent, and P 0.00 per cent. The reducing power (Hagedorn-Jensen method after hydrolysis with N HCl for 6 hours at 100° in a sealed tube) was equivalent to 61.8 per cent of glucose. While this preparation was not of a high degree of purity, it compared favorably with those described by Levene (9). Aqueous solutions of this substance were used in all the experiments.

First we studied the effect of extended boiling without otherwise modifying the procedure of Maughan *et al.* Some of these results are illustrated in Fig. 1 (section A). If the boiling was continued for $4\frac{1}{2}$ hours, the optical density of the chromogen when measured with Filter 565 increased in a rather regular manner in the case of all the substances investigated, but there was a definite lag in the color development in the case of mucoitinsulfuric acid as compared with glucuronic acid. That there was a relative lag also with mucin is evident from the fact that, while the optical density after 30 minutes of boiling was practically identical with that for glucuronic acid, there was a far greater development of color with mucin than with glucuronic acid or menthyl glucuronide. All curves tended to level off when boiling was continued for $3\frac{1}{2}$ to $4\frac{1}{2}$ hours. In a number of other experiments in which boiling was limited to $\frac{1}{2}$ to 1 hour, the lag in the color development with mucin was even more pronounced than in the experiments illustrated in Fig. 1. This phenomenon is unquestionably due to the fact that glucuronic acid as such is immediately available for the formation of chromogen, but when it is a constituent of mucin it must first be liberated in a free state. The behavior of menthyl glucuronide was very similar to that of glucuronic acid probably because it may be hydrolyzed with relative ease. The leveling off of the color development, observed with all the above substances when boiling is extended for $3\frac{1}{2}$ hours or more, can be explained by the fact that the chromogen formed with glucuronic acid and naphthoresorcinol under the conditions of our experiments reaches its maximum at about 4 hours. In this respect our observations confirm the earlier observations of Kapp (17) and Hanson *et al.* (19). Therefore it might be expected that with a sufficiently extended boiling time there should be no material difference in chromogen formation regardless of whether the glucuronic acid is available immediately as in the case of free glucuronic acid or whether it is only gradually liberated from mucin, provided the latter process is accomplished in a relatively short period of time.

The practical conclusions to be drawn from the above observations are that true recoveries of glucuronic acid may be expected with the procedure of Maughan *et al.* if boiling is extended to 4 hours or more, or that a shorter procedure might perhaps be developed if a certain degree of preliminary hydrolysis of mucin preceded the "coupling" with naphthoresorcinol. In order to study the latter possibility, another series of experiments was carried out, in which the aforementioned substances were subjected to preliminary hydrolysis with 3 N HCl in a boiling water bath for 270 minutes, this being followed by "coupling" with naphthoresorcinol for a fixed period of 30 minutes. This procedure will be further referred to as "separate" hydrolysis and coupling, as distinct from the term "simultaneous" hydrolysis and coupling referred to in the experiments already described. The results of the "separate" hydrolysis and coupling experiments are illustrated in Fig. 1 (section *B*). A progressive fall in color intensity with glucuronic acid and menthyl glucuronide occurred in these experiments, as was to be expected in view of the well known fact that glucuronic acid is rather easily destroyed by boiling with strong hydrochloric acid. Similar relations occurred with mucoitinsulfuric acid, while the curve for mucin tended to rise till the end of 60 minutes and then to remain more or less constant.

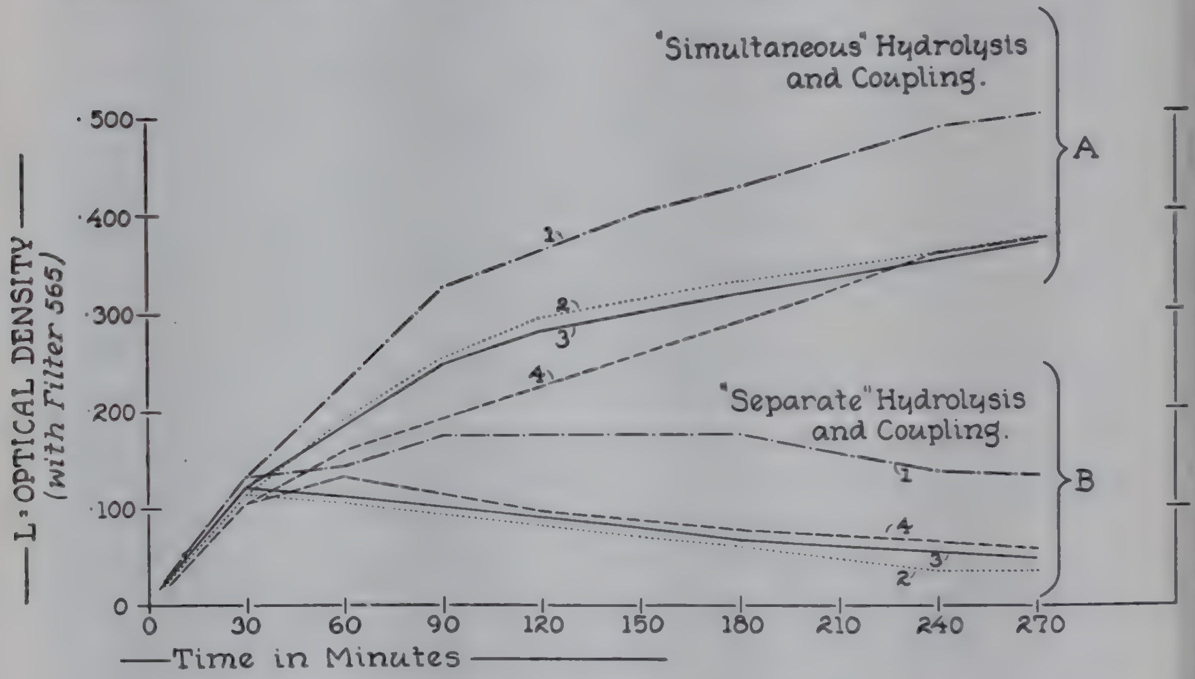


FIG. 1. Color development in the naphthoresorcinol reaction under various conditions of hydrolysis and coupling. *A*, Curve 1, gastric mucin (100 mg. per 100 ml.); Curve 2, glucuronic acid (1 mg. per 100 ml.); Curve 3, menthylglucuronic acid (2 mg. per 100 ml.); Curve 4, mucoitinsulfuric acid (10 mg. per 100 ml.). In *B*, the time includes 30 minutes coupling.

When the recovery of glucuronic acid from mucin and mucoitinsulfuric acid was calculated from the light densities in each of the experiments graphically illustrated in Fig. 1, the following results were obtained. In "simultaneous" hydrolysis and coupling experiments the recovery of glucuronic acid from mucin was considerably higher with 60 minutes than with 30 minutes boiling, and there was some tendency to a further increase if heating was extended further. The recovery of uronic acid from mucoitinsulfuric acid after 30 minutes of "simultaneous" hydrolysis and coupling was not uniform, but after 60 minutes it was uniform and not materially increased when the time of hydrolysis was extended. In experiments in which mucin and mucoitinsulfuric acid were subjected to preliminary "separate" hydrolysis, there was a steep increase in glucuronic acid recovery, especially from mucin. However, this should be regarded as only an apparent effect, due to the more rapid destruction of free glucuronic acid in the standards than in uronic acid, which is gradually liberated from mucin.

TABLE I

Estimates of Glucuronic Acid Content (Per Cent) in Mucoitinsulfuric Acid and Mucin under Various Conditions of Hydrolysis and Coupling

	Simultaneous hydrolysis and coupling, Filter 565			30 min. hydrolysis followed by 30 min. coupling	
	30 min.	60 min.	240 min.	Filter 565	Filters 565 and 400
Mucoitinsulfuric acid	11.6 ± 0.6*	9.4 ± 0.4	6.94 ± 1.08	11.0 ± 0.2	12.8 ± 0.3
Mucin	1.08 ± 0.04	1.21 ± 0.06	1.63 ± 0.36	1.34 ± 0.04	1.28 ± 0.03

The above figures in each case represent the results of six determinations which were started simultaneously with the same solutions.

* Standard deviation = $\pm\sqrt{\Sigma d^2/(n-1)}$ (Fisher).

These experiments therefore indicated that extended "separate" hydrolysis could not well be employed because of the deterioration of the standards. The highest recoveries of glucuronic acid from mucin and mucoitinsulfuric acid were obtained with the 30, 60, and 240 minute "simultaneous" procedure and also with the 30 minute preliminary "separate" hydrolysis. These procedures were subjected to more detailed study in order to determine more exactly the magnitude and also the reproducibility of the uronic acid recovery.

The results of a representative experiment are shown in Table I. The highest mean recovery of glucuronic acid from mucin was obtained with the "simultaneous" procedure of 4 hours duration, but the recoveries were not consistent (coefficient of variation 22 per cent). The next highest recoveries which were at the same time coincident with the smallest deviations (coefficient of variation 4 per cent) occurred when the mucin solutions were subjected to separate hydrolysis for 30 minutes prior to the 30 minutes "coupling."

This method, as will be demonstrated below, produced reliable results when applied to gastric mucin and pure canine gastric juice, but was found to be less satisfactory when applied to alkaline or neutral mucus. The hydrolysis of alkaline or neutral mucus with hydrochloric acid gave rise to large amounts of furfural, which interfered with the development of color with naphthoresorcinol. However, furfural and glucuronic acid produce entirely different colors, as may be seen from the absorption spectra (Fig. 5). Maximum absorption in the visible spectrum in the case of furfural takes place in the range of light transmitted by Rubicon Filter 400 and in the case of glucuronic acid in the band transmitted by Filter 565. Straight line calibration curves were obtained for the light densities measured at these two wave-lengths for both of these substances. Therefore conditions are present which permit corrections for extraneous furfural by application of the principles suggested by Knudson *et al.* (21) for a two-component color system. With calculations based on this principle, described below under "Method," more satisfactory recoveries were obtained from alkaline mucus and from gastric juice containing considerable proportions of mucus.

Method

Reagents—

1. Hydrochloric acid. Reagent grade, concentrated, sp. gr. 1.19.

2. Naphthoresorcinol.[1] 0.2 per cent filtered (No. 42 Whatman filter paper) aqueous solution; must be prepared immediately before analysis.

3. Ether. Merck, reagent, treated with 1 per cent ferrous sulfate to remove peroxides, washed with water until sulfate-free, and stored over anhydrous sodium sulfate.

4. Ethyl alcohol. 95 per cent.

5. Standard solutions of menthyl glucuronic acid and glucuronic acid. A stock standard solution of menthyl glucuronic acid is prepared so as to contain 4 mg. per ml., which may be kept in the refrigerator for not more than a month. A dilute standard solution (1:100) is made up at the time of analysis. The standard solution of glucuronic acid, containing 0.02 mg. per ml., must be prepared immediately before analysis.

Procedure

Hydrolysis—A sample of material (gastric juice or solution of mucin), containing 0.01 to 0.6 mg. of uronic acid (2 ml. for histamine gastric juice,

---

[1] Naphthoresorcinol was obtained from the Schwarz Laboratories, Inc., 202 East 44th Street, New York 17, New York.

1 ml. for sham feeding juice, and 0.2 ml. for mucus), is pipetted into special calibrated colorimeter tubes,[2] and the volume is adjusted with distilled water to 2 ml. Tubes with 2 ml. of a standard solution and 2 ml. of water (blank) are set up simultaneously. 1 ml. of concentrated hydrochloric acid is added, the contents being thoroughly mixed, and each tube is covered with a glass marble and placed in a boiling water bath for 30 minutes.

Coupling—The tubes are removed from the water bath, 2 ml. of naphthoresorcinol solution are added to each, and the tubes are thoroughly shaken. 1 ml. of concentrated hydrochloric acid is added. The contents of the tubes are mixed and the tubes are covered and placed again in the boiling water bath for a further period of 30 minutes.

Chromogen Extraction—The tubes are withdrawn and cooled in an ice bath for 10 minutes. 2 ml. of ethyl alcohol are added to the contents and mixed, followed by 15 ml. of ether. The tubes are stoppered with rubber stoppers[3] and shaken well by continuous, vigorous inversions for 30 seconds. The contents of the tubes are allowed to settle for 10 minutes, and the upper purplish colored layer is read in a special tube holder in the Evelyn colorimeter, with Filter 565 for the single filter procedure and Filters 565 and 400 for the two-filter procedure, after the blank (reagent) tube has been set at 100. The center setting should be no higher than 78. If it is any higher, the experiment must be discarded. As a rule the excessive color is due to deterioration of the naphthoresorcinol.

Calculations—For the one-filter procedure, L^{u}_{565}/L^{s}_{565} = mg. of glucuronic acid per 100 ml. of material if 0.02 mg. of glucuronic acid is used as standard and 2 ml. of material are taken.

For the two-filter procedure,

$$\frac{K^{f}_{565}\cdot L^{u}_{400} - K^{f}_{400}\cdot L^{u}_{565}}{K^{f}_{565}\cdot K^{s}_{400} - K^{f}_{400}\cdot K^{s}_{565}} \times \frac{100}{V} = \text{mg. glucuronic acid per 100 ml. material}$$

where V = ml. of material taken, or

$$\frac{0.86\cdot L^{u}_{400} - 2.70\cdot L^{u}_{565}}{-11.8} \times 50 = \text{mg. glucuronic acid per 100 ml. material}$$

---

[2] Special tube holder, No. 4626, of the Rubicon Company, Philadelphia 32, Pennsylvania.

[3] These stoppers must be first thoroughly washed with acetone until the washings are colorless. Before and after each set of determinations they are adequately rinsed with ether. These stoppers are used for these determinations exclusively. The suitability of any particular batch of stoppers is best indicated by the "center setting" of the blanks and the reproducibility of the calibration constants of the standard solutions.

if 2 ml. of material are taken and the calibration constants given below are used.

L^u_{400} = optical density of unknown with Filter 400
L^u_{565} = “ “ “ “ “ “ 565
L^s_{565} = “ “ “ standard “ “ 565

Calibration Constants—K = (optical density)/(mg. per aliquot). With Filter 565, $K^f_{565} = 0.86$ for furfural and $K^g_{565} = 4.72$ for glucuronic acid. With Filter 400, $K^f_{400} = 2.70$ for furfural and $K^g_{400} = 1.09$ for glucuronic acid.

TABLE II

Reproducibility of Calibration Constants and Estimates of Glucuronic Acid Content in Various Substances with Proposed Procedures

Filter No.	Substance	n	Calibration constants*			Glucuronic acid		
			K	S.D.	Coefficient of variation	Per cent	S.D.	Coefficient of variation
565	Glucuronic acid	22	5.30	0.45	8.5			
	Menthylglucuronic acid	218	2.82	0.15	5.3	102.0†	4.5	4.4
	Mucoitinsulfuric acid	45	0.69	0.09	13.0	13.3	1.7	12.8
	Mucin	29	0.067	0.006	8.8	1.37	0.09	6.6
	Canine gastric juice	18‡	0.30	0.02	6.0	2.63	0.02	0.8
565 and 400	Mucoitinsulfuric acid	45				13.6	1.2	8.8
	Mucin	29				1.22	0.09	7.4

* K = (optical density)/(mg. or ml. per aliquot); S.D. = $\pm\sqrt{\Sigma d^2/(n-1)}$; coefficient of variation = (S.D./mean) × 100.

† From twenty-two determinations.

‡ Triplicate determinations of six dilutions of the same specimen, obtained after sham feeding from a dog with a gastric fistula and esophagotomy.

Since no preparations of gastric mucin of generally accepted purity, which could be employed as a standard, were described, the conversion of glucuronic acid values to those of mucin cannot be justified. However, in some of our studies (22), when such a conversion was deemed necessary for clarity of presentation, a conversion factor derived from the mean values of glucuronic acid content in our best preparation of mucin presented in Table II was used.

Absorption Spectra and Recovery Curves

Using the procedure described above, we have compared the absorption spectra of gastric mucin, mucoitinsulfuric acid, glucuronic acid, menthylglucuronic acid, furfural, and gastric juice of different degrees of purity.

From Fig. 2 it may be seen that the absorption curves for glucuronic acid and its derivatives and for pure canine gastric juice, such as that obtained, for example, at the height of the secretion produced by sham feeding, are identical, maximum absorption with Filter 565, while the curve for furfural (Fig. 5) is utterly different. These observations justify the use of single Filter 565 for pure gastric juice. The color development of the above reference substances and pure gastric juice follows Beer's law for optical densities measured with Filter 565 (Fig. 3). Straight line curves for glucuronic acid recovery for gastric mucin and mucoitinsulfuric acid were always obtained with the one-filter procedure, as illustrated by Fig. 4. The recovery of glucuronic acid in the experiments in which mucin was added to canine gastric juice was equally satisfactory.

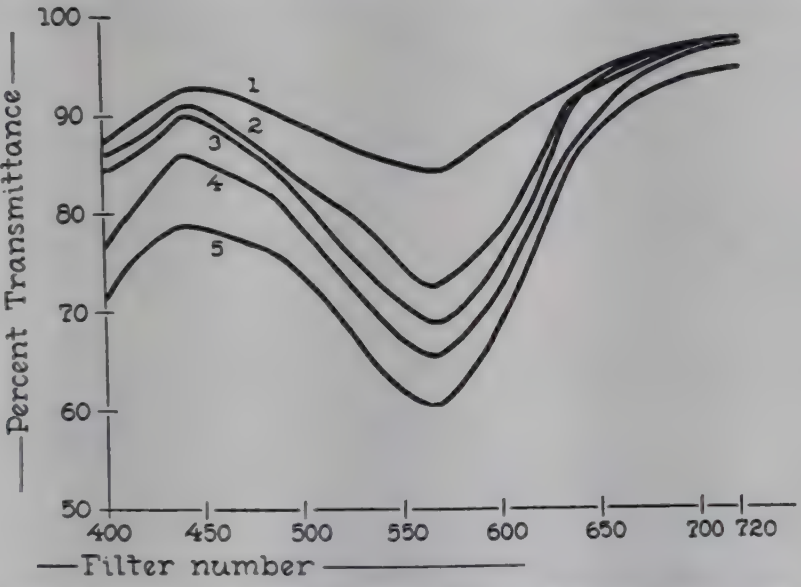


FIG. 2. Light absorption curves. Curve 1, pure gastric juice, sixth 15 minute fraction after sham feeding, pH 0.91, chlorides 162 milliequivalents per liter, pepsin 77 Mett units; Curve 2, barium salt of mucoitinsulfuric acid (10 mg. per 100 ml.); Curve 3, glucuronic acid (6 mg. per 100 ml.); Curve 4, gastric mucin (100 mg. per 100 ml.); Curve 5, menthylglucuronic acid (10 mg. per 100 ml.).

Reliability of Method

The reproducibility of the one-filter procedure may be considered adequate in view of the values for standard deviations and coefficients of variation (Table II). In experiments performed over a period of 2 years in a routine manner with different batches of reagents and with a wide range of concentrations of all the substances studied, the coefficient of variation ranged from 5.6 to 12.8 per cent. Much greater uniformity was obtained in individual experiments even on a very large scale, as may be seen from Table I (last section), where the coefficients of variation for

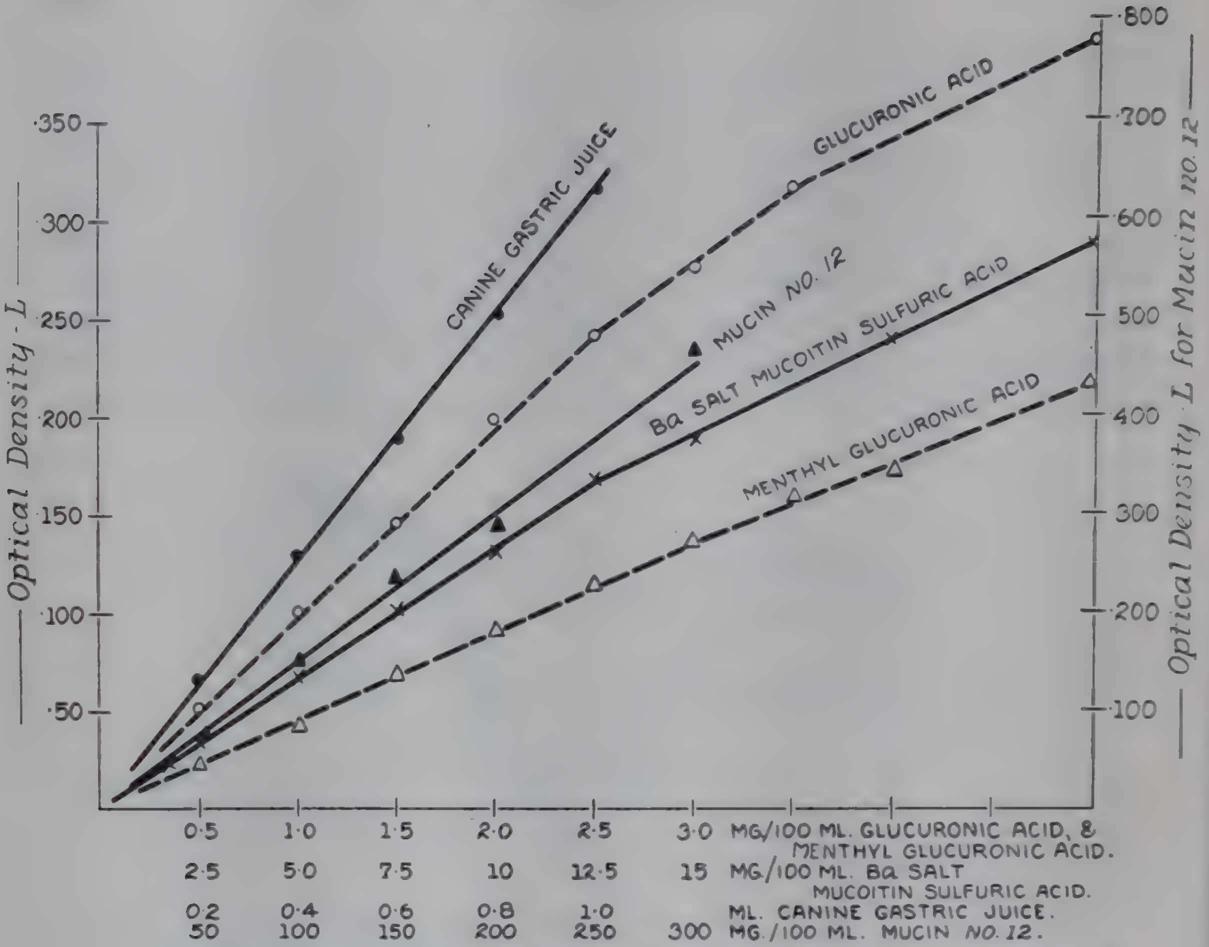


FIG. 3. Calibration curves with one-filter procedure in terms of optical densities

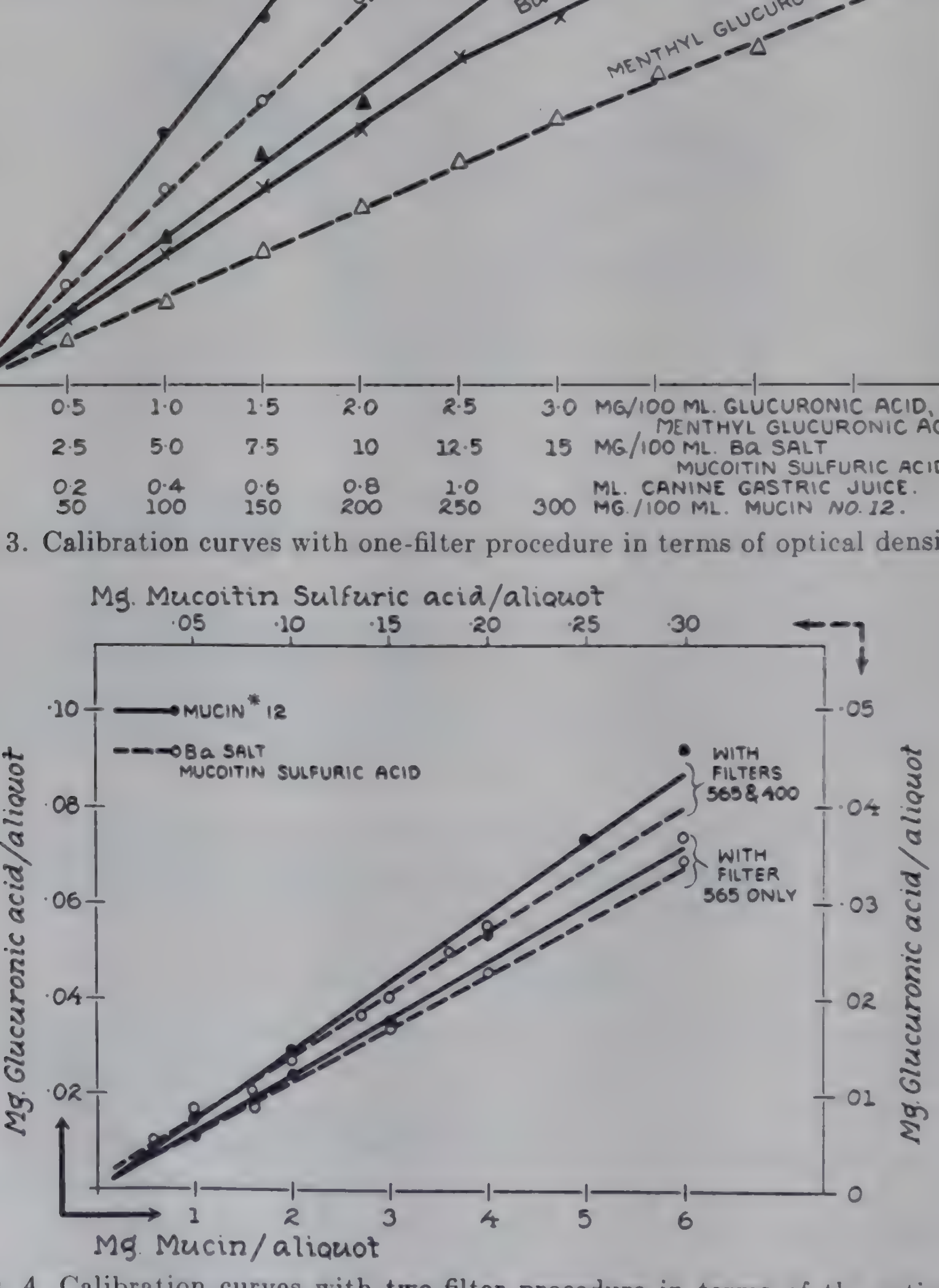
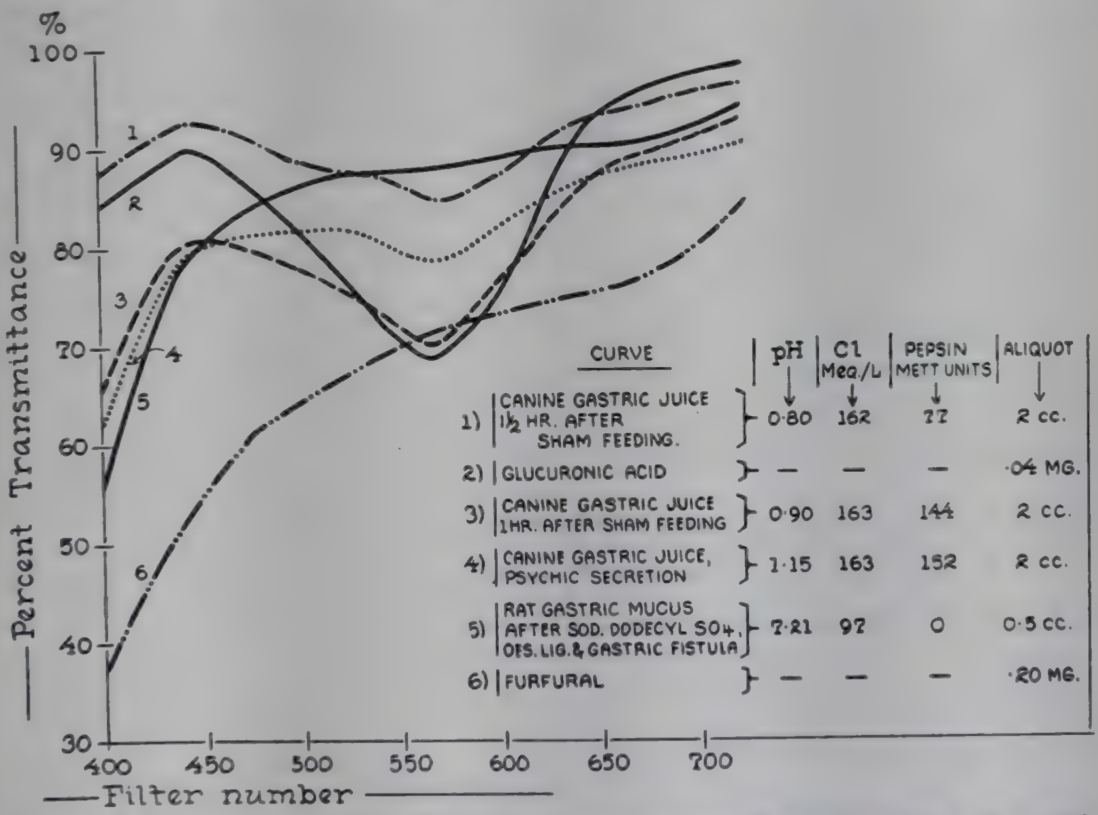


FIG. 4. Calibration curves with two-filter procedure in terms of the estimated glucuronic acid content.

our routine procedure were only 1.8 per cent for mucoitinsulfuric acid and 3.0 per cent for mucin.

The absorption curves obtained with gastric juice which is not quite pure, particularly if mixed with mucus, have characteristics common to both glucuronic acid and furfural (Fig. 5). Many specimens of mucus (Fig. 5, Curve 5), especially those obtained from rats, and the first specimens of acid gastric juice collected in experiments on gastric fistula dogs (Fig. 5, Curve 4), which always contain a considerable amount of admixed mucus, give absorption curves closely resembling those of furfural. It was for such specimens that we found it necessary to use our two-filter procedure. With mucin and mucoitinsulfuric acid, the reproducibility of the two-filter procedure was comparable to that obtained with the one-filter modification, as may be seen from the values for standard deviations and coefficients of variation in Table II. There was a slight difference in the magnitude of glucuronic acid recovery, but this does not seem to be significant. Straight line glucuronic acid recovery curves for mucin and mucoitinsulfuric acid resulted with both modifications (Fig. 4). Comparable recoveries with both modifications were obtained in experiments in which known amounts of mucin were added to canine gastric juice. However, the available evidence seems to indicate that truer recoveries



CURVE	pH	Cl Meq./L	PEPSIN METT UNITS	ALIQUOT
1) CANINE GASTRIC JUICE 1½ HR. AFTER SHAM FEEDING.	0·80	162	77	2 cc.
2) GLUCURONIC ACID	—	—	—	·04 MG.
3) CANINE GASTRIC JUICE 1 HR. AFTER SHAM FEEDING	0·90	163	144	2 cc.
4) CANINE GASTRIC JUICE, PSYCHIC SECRETION	1·15	163	152	2 cc.
5) RAT GASTRIC MUCUS AFTER SOD. DODECYL SO4, OES. LIG. & GASTRIC FISTULA	7·21	97	0	0·5 cc.
6) FURFURAL	—	—	—	·20 MG.

FIG. 5. Comparison of the light absorption curves of various types of gastric secretion with those of glucuronic acid and furfural.

of mucin are obtained from alkaline gastric mucin and from not quite pure gastric juice with the two-filter than with the one-filter procedure. However, this evidence is not conclusive and further study is necessary.

Source of Uronic Acid in Gastric Juice

Mucoprotein should in all probability be regarded as practically the only source of the uronic acid that is liberated on acid hydrolysis of pure canine gastric juice. This is evident from the results of experiments in which uronic acid was determined in the filtrates after the removal of protein by several procedures; *viz.*, precipitation with acetone, basic lead acetate, and aluminum hydroxide. Acetone, under the experimental conditions obtaining, has been shown to precipitate all protein from freshly secreted canine gastric juice (23); precipitation with lead acetate at pH 6.4 to 6.8 has been generally regarded as one of the few specific precipitation procedures for mucoproteins, and we have found that aluminum hydroxide precipitates both the pepsin and the mucin of gastric juice quantitatively. Results obtained with gastric juice secreted in response to sham feeding are shown in Table III. Not more than 15 per cent, and in the majority of these experiments only 2 to 5 per cent, of the total glucuronic acid was recovered from these filtrates. Virtually all the glucuronic acid of the gastric juice (with a mean of 98.4 per cent) was recovered after the crystalloids had been removed by overnight dialysis. Similar results were obtained in several dialysis experiments with alkaline mucus collected from dogs with a gastric fistula and esophagotomy.

It is known that pepsin in an acid medium slowly digests mucoproteins, and peptic digestion has in fact been extensively used in the past as a preliminary step in isolating the carbohydrate complex of mucoproteins, especially chondroitinsulfuric acid. In our experiments with protein precipitants, the specimens of gastric juice were subjected to analysis not immediately after collection but after standing at 5° for 3 to 24 hours. Some degree of hydrolysis may therefore have taken place with the splitting off of mucoitinsulfuric acid and its derivatives, and this may account for the small amounts of glucuronic acid found in the deproteinized filtrates. Therefore it is justifiable to conclude that the uronic acid of the gastric juice is derived predominantly or perhaps even exclusively from its protein constituents.

Mucin Content of Gastric Secretion under Different Conditions of Stimulation

The method described above was instrumental in establishing several physiologically important facts concerning the quantitative aspects of mucin secretion in relation to the nature of the stimulation. This part of the study will be published in detail elsewhere (24). However, several observations should be stressed here. Table IV shows that there were exceedingly wide variations in the mucin concentration of different types

TABLE III

Partition of Uronic Acid Derivatives in Canine Gastric Juice

Specimen No.	pH	Chloride	Pepsin, Mett units	Total glucuronic acid	Non-dialyzable fraction*	In filtrates* after precipitation with		
						Lead subacetate	Aluminum hydroxide	Acetone
		m.eq. per l.		*mg. per 100 ml.*				
1	0.78	160	85	2.90	89 (8)	5 (2)		
2	0.96	154	31	1.56		10 (2)	6 (4)	
3	0.91	167	92	1.44		2 (2)	2 (12)	8 (2)
4	0.90	164	98	1.35	99 (12)	9 (2)		10 (2)
5	0.98	165	117	0.98	115 (4)	5 (2)	5 (8)	15 (2)
6	0.94	171	41	0.68			7 (12)	
7	1.00	172	23	0.61		0 (2)	10 (2)	

* Expressed in percentage of total glucuronic acid.

The figures in parentheses represent the number of experiments with respective specimens.

Gastric juice or mucus was introduced into cellophane tubing (Nojax, Visking Corporation) and allowed to dialyze against tap water, distilled water, or physiological saline.

For precipitation with lead subacetate (Merck) the "free" acid in the gastric juice was neutralized with a calculated amount of 1.0 N NaOH, and 0.1 N NaOH was added to make the pH 6.8 to 7.0. 10 per cent lead subacetate was then added from a burette until no further precipitation was obtained. After standing in the refrigerator overnight, the sample was centrifuged and filtered. The pH of the filtrate ranged from 6.4 to 6.8.

For precipitation with colloidal aluminum hydroxide two procedures were used which gave comparable results. (1) The "free" acidity of the aliquot of gastric juice was neutralized with a calculated amount of N NaOH, and 0.1 to 0.2 volume of colloidal aluminum hydroxide was added. The solution was left in the refrigerator overnight and the supernatant was then filtered through No. 40 Whatman filter paper. (2) 0.25 volume of 10 per cent aluminum chloride was added to gastric juice and the necessary amount of NaOH to make the pH 5.8 to 6.9 (as previously determined in a separate sample) was added rapidly with vigorous mixing. After the solution had stood overnight in the refrigerator, the supernatant was filtered through No. 40 Whatman filter paper.

Precipitation with acetone was carried out as previously described (23).

of gastric secretion which were equivalent to 0.06 to 28.6 mg. per cent of glucuronic acid. The lowest concentrations were found in the secretion following histamine administration, the highest in pure alkaline mucus secreted by the stomach of a fasting animal, while those for gastric juice obtained after sham feeding were in the middle range. These wide variations are important methodologically, since (*a*) they should be considered in selecting the size of the aliquots to be taken for mucin determination, and (*b*) they indicate that the magnitude of error inherent in the method described is sufficiently small not to jeopardize its value in physiological studies.

TABLE IV

Mucin Concentration (As Glucuronic Acid) in Gastric Secretion under Various Conditions of Stimulation

No. of experiments	Dog	Type of secretion	Rate	pH	Pepsin, Mett units	Glucuronic acid	
			ml. per hr. per kg.			*mg. per 100 ml.*	*mg. per hr. per kg.*
During same experiment	A	Fasting	0.53	8.02	0	14.1	0.07
		Sham feeding	12.5	1.08	58	2.6*	0.32
		" " and histamine	12.5	0.94	3	0.34*	0.04
5		Spontaneous mucus	0.52	8.20	0	7.56	0.04
4		Sham feeding, total secretion	9.3	1.03	33	1.84*	0.18
1		Sham feeding, height of secretion	10.2	0.93	31	0.61*	0.06
2		After histamine	14.0	0.92	<1	0.06	<0.01
5	B	Sham feeding, total secretion	>8.8	0.92	85	1.50*	>0.13
2		Spontaneous mucus	0.26	7.80	0	6.75	0.02
1		+ atropine	0.14	8.30	0	28.6	0.04
2		After sodium dodecyl sulfate	4.6	7.70	0	13.4	0.62
1		+ atropine	6.6	8.70	0	23.5	1.55

* Mucin in solution.

DISCUSSION

Glucuronic acid is regarded as a component of mucoitinsulfuric acid, the characteristic prosthetic group of gastric mucin (9, 13). Consequently we selected glucuronic acid or menthyl glucuronide or both as reference substances and expressed the results of our analyses in terms of glucuronic acid. The absorption curves obtained by us for glucuronic acid or its derivatives were quite different from those which we obtained for furfural. This provides further confirmation of the view, first expressed by Mandel and Neuberg (25), that furfural is not responsible for the characteristic with HCl, as has been postulated by Tollens and many other investigators. The absorption curves produced by mucin and by pure gastric juice were strikingly similar to the curves produced by glucuronic acid, while those for alkaline gastric mucus showed features common to the curves for both glucuronic acid and furfural. This indicates that any method based on the determination of furfural alone cannot be utilized for the estimation of mucin. Furthermore, furfural formed from sources other than mucin was found to be detrimental to the estimation of mucoproteins by the naphthoresorcinol method if the calculations were based solely on the light density read at the band of maximum absorption with Filter 565. Only correcting for extraneous furfural made possible by application of the two-filter procedure seemed to make the naphthoresorcinol method more specific and to result in satisfactory recoveries from pure and not too heavily contaminated gastric juice.

We are aware that the method proposed here for the estimation of mucin in gastric juice does not meet the most exacting requirements of quantitative analysis, but to our knowledge it is the only method described so far which has been subjected to an exhaustive series of tests of reliability and has given results reproducible within 10 per cent. We believe that this method will be valuable in the solution of many important problems in the physiology and pathology of the gastric glands.

SUMMARY

A quantitative method for the estimation of mucin in the gastric juice and gastric contents has been developed, based upon the determination of glucuronic acid, a characteristic component of the prosthetic group of mucoproteins. The uronic acid is determined by a photoelectric-colorimetric method by the use of Tollens' naphthoresorcinol reaction, as modified by Maughan, Evelyn, and Browne (16), after preliminary acid hydrolysis of the material. With gastric mucin and its derivatives the resulting color is a two-component color system with two maxima of light absorption obtained with Filters 565 and 400. The former band is characteristic for uronic acid itself and the latter for furfural, which may be derived either from uronic acid or from other substances, as in the case of gastric mucus or not quite pure gastric juice.

Reproducible results were obtained for mucin, mucoitinsulfuric acid, and pure gastric juice from the light densities determined in an Evelyn photoelectric colorimeter with Filter 565 alone, glucuronic acid or menthyl glucuronide being used as a standard. For mucus and contaminated gastric juice, it was necessary to determine light densities with Filters 565 and 400 by calculations based on the principles developed by Knudson, Meloche, and Juday (21). This procedure gave reproducible results also with mucin and mucoitinsulfuric acid. Fractionation experiments with various protein precipitants and dialysis demonstrated that only insignificant amounts of uronic acid were present in the protein-free fractions of canine gastric juice. It is probable that these small quantities may be derived from products of the enzymatic hydrolysis of mucin.

The concentration of mucin varied greatly in different types of gastric secretion. It was highest in alkaline mucus secreted either spontaneously or in response to intragastric instillation of sodium dodecyl sulfate and lowest in gastric secretion provoked by histamine administration. The concentration of mucin in the juice secreted in response to sham feeding was much higher than that of the gastric juice following histamine administration.

Addendum—Recently, after this study had been virtually completed, a new and specific color reaction for hexuronic acid with carbazole was reported by Dische (26), who claimed it to be suitable for the quantitative determination of hexuronic acid in various uronides and possibly also in some biological fluids. However, the presence of excessive amounts of protein in proportion to uronic acid appeared to jeopardize the results. We have attempted to explore the possibility of the application of this reaction to the determination of mucin in gastric secretion. The color development with glucuronic acid and menthylglucuronic acid (1 and 2 mg. per cent), when measured by the Evelyn colorimeter with Rubicon light Filter 520, was reproducible with a 12 per cent coefficient of variation, and the recovery of glucuronic acid from menthyl glucuronide was within 1 per cent of the theoretical value. The absorption spectra produced with our preparations of mucoitinsulfuric acid and gastric mucin were identical with that of glucuronic acid. A straight line recovery curve for hexuronic acid was obtained in experiments with gastric mucin (in a range of 100 to 400 mg. per cent) and mucoitinsulfuric acid (in a range of 3.0 to 50 mg. per cent) solutions. The uronic acid content, determined by the carbazole method, was 1.55 ± 0.08 per cent for mucin and 11.1 ± 1.1 per cent for mucoitinsulfuric acid. The reproducibility of the carbazole method therefore approximates that of our naphthoresorcinol method. In view of the greater simplicity of the carbazole method, and especially in view of its specificity, it deserves to be explored further with the purpose of applying it to the estimation of mucin in gastric secretion and the gastric contents.

BIBLIOGRAPHY

1. Komarov, S. A., Siplet, H., and Shay, H., *Federation Proc.*, **6**, 144 (1947).
2. Babkin, B. P., Secretory mechanism of the digestive glands, New York (1944).
3. Dienst, C., *Deut. Arch. klin. Med.*, **171**, 52 (1931).
4. Mielke, H., Dissertation, Greifswald (1932).
5. Baltzer, F., *Biochem. Z.*, **264**, 28 (1933); *Arch. Verdauungskr.*, **56**, 35 (1934).
6. Burger, G. N., Hartfall, S. J., and Witts, L. J., *Guy's Hosp. Rep.*, **83**, 497 (1933).
7. Mahlo, A., *Fortschr. Therap.*, **11**, 16, 84 (1935).
8. Glass, J., *Klin. Wochschr.*, **17**, 1802 (1938).
9. Levene, P. A., Hexosamines and mucoproteins, Monographs on biochemistry, New York and Toronto (1925).

10. López-Suárez, J., *Biochem. Z.*, **56**, 167 (1913).
11. Levene, P. A., and López-Suárez, J., *J. Biol. Chem.*, **25**, 511 (1916); **26**, 373 (1916); **36**, 105 (1918).
12. Meyer, K., Smyth, E. M., and Palmer, J. W., *J. Biol. Chem.*, **119**, 73 (1937).
13. Wolfrom, M. L., Weisblat, D. I., Karabinos, J. V., McNeely, W. H., and McLean, J., *J. Am. Chem. Soc.*, **65**, 2077 (1943).
14. Komarov, S. A., *J. Biol. Chem.*, **109**, 177 (1935).
15. Tollens, C., *Z. physiol. Chem.*, **56**, 115 (1908); **61**, 95 (1909).
16. Maughan, G. B., Evelyn, K. A., and Browne, J. S. L., *J. Biol. Chem.*, **126**, 567 (1938).
17. Kapp, E. M., *J. Biol. Chem.*, **134**, 143 (1940).
18. Dziewiatkowski, D. D., Thesis, University of Michigan (1944).
19. Hanson, S. W. F., Mills, C. T., and Williams, R. T., *Biochem. J.*, **38**, 274 (1944).
20. Williams, R. T., *Biochem. J.*, **34**, 272 (1940).
21. Knudson, H. W., Meloche, V. W., and Juday, C., *Ind. and Eng. Chem., Anal. Ed.*, **12**, 715 (1940).
22. Shay, H., Komarov, S. A., Siplet, H., and Fels, S. S., *Science*, **103**, 50 (1946). Shay, H., Komarov, S. A., Siplet, H., and Gruenstein, M., *Am. J. Digest. Dis.*, **14**, 99 (1947).
23. Komarov, S. A., *J. Lab. and Clin. Med.*, **23**, 822 (1938).
24. Komarov, S. A., Shay, H., and Siplet, H., *Federation Proc.*, **7**, 67 (1948).
25. Mandel, J. A., and Neuberg, C., *Biochem. Z.*, **13**, 148 (1908).
26. Dische, Z., *J. Biol. Chem.*, **167**, 189 (1947).

THE ABSORPTION SPECTRA OF VISUAL PURPLE OF THE SQUID AND ITS BLEACHING PRODUCTS*

By ALFRED F. BLISS

(*From the Department of Physiology, Tufts College Medical School, Boston*)

(Received for publication, June 28, 1948)

The eyes of some invertebrates, notably the cephalopods, are superficially remarkably similar to those of vertebrates. The rod cells of both types of retinas contain reddish pigments loosely termed "visual purple" by earlier workers. In more recent years it was assumed that the invertebrate rod pigment (1) was probably inert melanin, and that only in vertebrates could the rod pigment be clearly correlated with vision.

Our knowledge of retinal photochemistry is based almost entirely on the vertebrate visual purple, rhodopsin. This pigment bleaches in the light with the release of a series of poorly defined intermediates (2–6), the first of which was appropriately named transient orange by Lythgoe and Quilliam (2). Transient orange is relatively stable at 0° but decomposes in a few minutes at room temperature, forming a reversibly pH-sensitive pigment called indicator yellow by Lythgoe (3). Indicator yellow can be exacted from the retina in a petroleum ether-soluble form by 70 per cent alcohol (4). In non-alkaline solutions above pH 4 indicator yellow decomposes (6) with the release of retinene (7). The decomposition is probably reversible, since the addition of synthetic retinene (vitamin A aldehyde) to solutions of various proteins and amino acids yields colored complexes resembling indicator yellow (8). An extractable retinal factor (6) brings about the conversion of the bleaching products of rhodopsin to vitamin A. In the dark-adapted organism, rhodopsin is regenerated from vitamin A.

In recent years the study of invertebrate retinas has been resumed, and with Wald's discovery (9) that the squid retina contained retinene it appeared likely that progress in this field would parallel that of vertebrate vision. However, there was no evidence of a photosensitive retinene precursor or visual purple in squid retinal extracts. In 1943 such a pigment was reported (10), but, surprisingly, it was photosensitive only in the presence of dilute denaturants such as alcohol or formalin. Nevertheless, a relation to the visual processes was suggested by the fact that the bleaching of the formalin-treated squid retina was accompanied by the release of large quantities of retinene. The difference between the absorption spectrum of the unbleached and bleached pigment in aqueous saponin or digitonin

---

* Aided by a grant from the American Philosophical Society and the Charlton Fund of Tufts College Medical School.

roughly resembled that of vertebrate rhodopsin and the visibility spectrum of the living cephalopod eye (11).

The fact that the squid contains a rich store of a photostable or nearly photostable "visual purple" is of interest in view of the long accepted datum that stimulation of the retina by light is invariably accompanied by the bleaching of a visual pigment. Hartline, Milne, and Wagman (12 have recently reported an interesting physiological study of the retina of the horseshoe crab *Limulus*, which like the squid contains a formaldehyde-sensitizable pigment. It was found that the number of quanta absorbed by the visual pigment (13) in bringing about a constant electric response of the eye was not constant but was greater when the sensitivity of the eye was decreased by light adaptation. This suggests that the amount of unbleached visual pigment was greater than that necessary to account for the decreased sensitivity of the light-adapted eye. Thus the bleaching of the visual pigment of *Limulus* appears insufficient to explain the decreased sensitivity of the light-adapted eye. Nevertheless, the kinetics of light and dark adaptation of the *Limulus* eye appear to be otherwise identical with those of vertebrate eyes (14).

It is possible, therefore, that a more detailed study of these curious invertebrate retinal pigments may prove important to the photochemistry of vertebrate as well as invertebrate vision. The following questions are considered in this paper: (1) What is the true absorption spectrum of the formalin-sensitizable pigment of the squid, in the absence of the large quantities of melanin present in earlier extracts? (2) Are the intermediate pigments of the vertebrate visual cycle, such as indicator yellow, formed when the squid "visual purple" is bleached?

EXPERIMENTAL

Preparation of Melanin-Free Retinal Extracts—Vertebrate visual purple can be extracted from the retinal rod cells by mild detergents such as aqueous digitonin. However, such extracts may contain large amounts of light-absorbing impurities such as proteins, melanin, and oil droplets. These impurities can be greatly reduced by peeling off the melanotic screening layer either mechanically or by Saito's method: centrifugation of retinal suspensions in concentrated sucrose (15). In the latter case, th retinal rods centrifuge upwards and the melanin downward. Hardenin the retina in alum (4 per cent potassium aluminum sulfate) or, less drasti cally, in a buffer at about pH 4.5 is also of great value in reducing the solu bility of both proteins and melanin. Oil droplets are removed by washin with non-polar solvents such as petroleum ether.

Unfortunately, the melanin of the squid retina, although forming distinct posterior screening layer, is partly within the retinal rod laye nd therefore cannot be peeled off. However, a nearly melanin-free, right red layer could be obtained by blotting the anterior layer on filter aper. The red pigment was rapidly bleached by dilute formalin in the ght, or by chloroform in the dark, and the bleaching was accompanied by he release of retinene. The effect of formalin was reversible, since rinsing the retinas in sea water abolished the light sensitivity.

An attempt was made to purify the red pigment by pretreatment of the retinas with alum, but destruction of the pigment resulted. However, a combination of Saito's method of sucrose homogenization and washing with an acid buffer proved successful and less painstaking than the mechanical separation described above. Squid retinas were dissected in ordinary daylight and preserved at $-20°$ until used. Two medium sized retinas were then ground about a minute in a Potter homogenizer with 5 ml. of a solution containing 2 gm. of sucrose and 3 ml. of water. Centrifugation yielded a black bottom layer and a red top layer, separated by opalescent red fluid. The top layer and red fluid were mixed, diluted with an equal volume of water, and centrifuged. The sediment was washed first with 0.1 M Na_2HPO_4 and then with cold phosphate-citrate buffer, pH 4.5. From this point on all operations were conducted as rapidly as possible in the cold. The red sediment was extracted for 2 minutes at 0° with 0.5 ml. of 3 per cent aqueous digitonin. Centrifugation yielded a clear red solution which was pipetted into a 2 mm. cell previously cooled with its holder in the freezing compartment of the refrigerator. The absorption spectrum was then measured with a potentiometric spectrophotometer (16). The spectrum, corrected for the absorption of the cell and solvent is shown by Curve 1 of Fig. 1. The absorption peak is at about 495 mμ, slightly but distinctly displaced toward the blue from that of vertebrate rhodopsin, which is 502 mμ. The absorption spectrum of frog rhodopsin, prepared from alum-pretreated retinas, was determined on the same instrument and is presented also in Fig. 1 (Curve 2).

Stability—The squid pigment was quite stable before extraction, and squid from local fish markets gave high yields, even though their retinas were thoroughly distintegrated by the time they were bought. However, the case was quite the opposite in aqueous digitonin, the absorption peak declining about 25 per cent in 30 minutes at pH 6.1 and 25.0°. The decomposition was not due to the light, since it was not accelerated by a 10 minutes exposure to a 100 watt light at 6 inches distance. 1 minute of such illumination brought about nearly complete bleaching of frog visual purple.

Photosensitivity of the squid "visual purple" was readily induced by addition of formaldehyde to make a concentration of 0.5 M. Since the thermal decomposition of the pigment was also increased, to about double the normal rate, rapid measurement of the spectrum was obligatory. The effect of light on a formalized extract is shown in Fig. 2 and indicates a photosensitivity about 5 per cent that of vertebrate visual purple.

Squid "Indicator Yellow"—Photic or thermal decomposition of rhodopsin results in the liberation of indicator yellow, which in mildly acid buffers fades in the course of about 3 hours. It was of interest to determine whether indicator yellow played a similar rôle as an intermediate in the decomposition of the photostable retinene precursor of the squid. The following procedure, used by Lythgoe for the demonstration of vertebrate indicator yellow, was followed. 0.01 ml. of 10 N NaOH was added to 0.5 ml. of an extract of squid "visual purple," causing bleaching of the pigment in a few seconds, as shown by Curve 2 of Fig. 3. After 2 hours at room temperature, 0.02 ml. of concentrated HCl was added. The extract quickly became deep yellow, due to the development of an absorption peak at 445 mμ. These changes are very similar to those shown by frog indicator

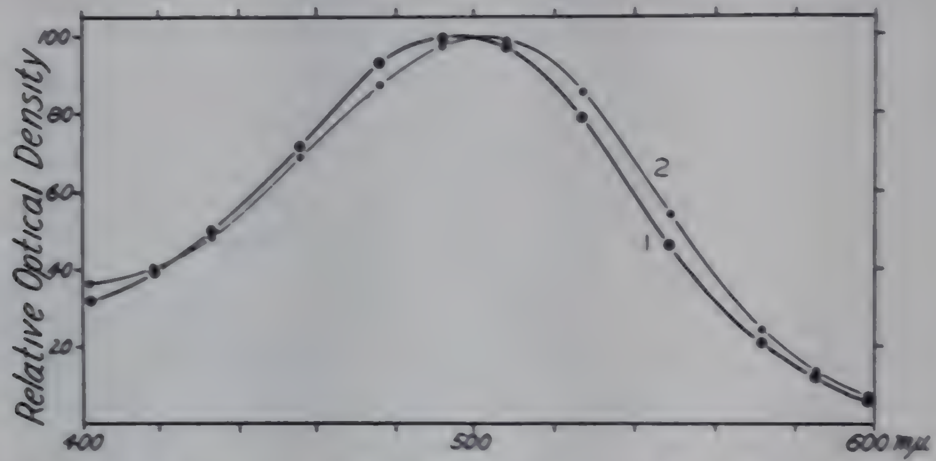


FIG. 1. Curve 1, absorption spectrum of purified "squid visual purple;" Curve 2, purified frog visual purple.

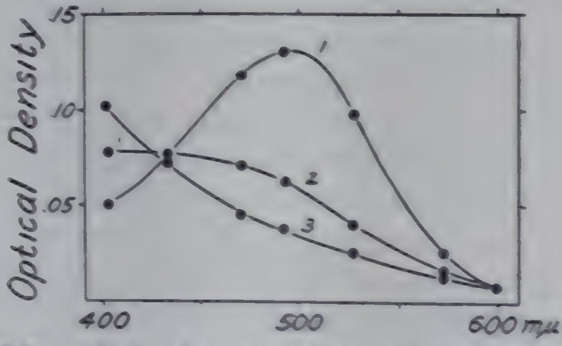


FIG. 2. Bleaching of "squid visual purple" by light in 0.5 M formaldehyde. Curve 1, before exposure to 100 watt light at 1 foot at 25°; Curve 2, after 10 minutes in light; Curve 3, after 20 minutes in light.

yellow. The absorption spectrum of the acid pigment was quite stable at room temperature, as reported by Lythgoe for acid indicator yellow below pH 4.

Pigments having indicator properties and soluble in petroleum ether containing polar solvents have been extracted from squid as well as from vertebrate retinas. In the case of the squid, two retinas were soaked in 0.1 M Na_2CO_3 for 15 minutes, ground with acetone, and extracted from 50 per cent acetone by petroleum ether. The residue obtained by drying the petroleum ether was colorless but became bright orange on addition of acid chloroform, due to an absorption peak at about 475 mμ.

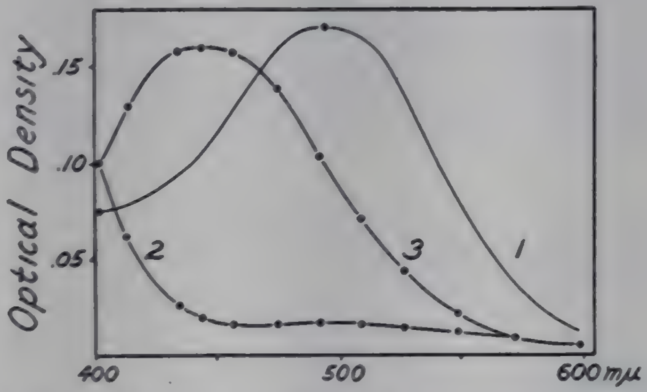


Fig. 3. Effect of pH on bleaching products of "squid visual purple." Curve 1, density at 402 and 495 mμ before bleaching; Curve 2, after adding NaOH; Curve 3, after adding HCl.

DISCUSSION

It is believed that the squid "visual purple" has been prepared in as pure a state as the best extracts of vertebrate visual purple, because the methods employed are known to reduce the solubility of protein and melanin impurities in such extracts to nearly negligible levels. Melanin (17) is particularly dangerous because of its high extinction coefficient. However, its solubility at pH 4.5 is so low that retinal material containing relatively enormous amounts of melanin yields extracts only slightly less pure than those prepared from demelanized homogenates.

A detailed comparison of the vertebrate and invertebrate "visual purples" may be of value in an attempt to assign a rôle in the visual cycle to the squid "visual purple." Such a comparison is presented in Table I.

The bleaching by light of the formalin-sensitized squid "visual purple" serves merely as a device whereby it is possible to demonstrate a latent photosensitivity of the red pigment. It should not be inferred that formaldehyde itself is present in the living retina, or that formaldehyde is unique in this action. Furthermore, the most recent measurements (18) on living squid retinas show no effect of light on their retinene precursor. Because of this unusual light stability, a specific name, cephalopsin, has been proposed for the visual purple of the squid.

It thus appears that the view of the nineteenth century workers, that the squid "visual purple" was photochemically active, may have been closer to the mark than more recent workers may be willing to concede. However, it does not yet appear possible to frame a more specific conclusion than the following (10):

> "Just how related all this is to the vision of the squid is hard to say. It may merely be that the normal squid photopigment is relatively light-stable, and that the formalin treatment renders it light-unstable. In that case serious consideration must be given to the possibility that the bleaching of vertebrate visual purple is a specialization and that the absence of bleaching, as in photosynthesis and photodynamic action, may have no direct bearing on the efficiency of a visual pigment."

TABLE I

Properties of Purified "Visual Purples"

	Frog	Squid
Absorption peak, mμ	502	495
Thermal stability	High	Low
Photic "	Low	High
Photosensitizers	H_2O	H_2O + denaturants
Effect of polar solvents	Bleaching	Bleaching
Intermediates of bleaching	Indicator yellow	Indicator yellow
Acid digitonin, mμ	445	445
Basic "	Colorless	Colorless
End-product of bleaching	Retinene	Retinene

SUMMARY

1. The squid retina contains a relatively light-stable red pigment with maximum absorption at 495 mμ, which can be obtained in a nearly melanin-free state by centrifugation of retinal homogenates in 40 per cent sucrose. The pigment can be extracted from the non-melanoid fraction at pH 4.5 by aqueous digitonin.

2. The pigment is bleached by light in the presence of dilute denaturants or in the dark by higher concentrations, yielding a colorless product in alkaline solutions. The colorless product becomes deep yellow in acid solutions.

3. The bleaching process releases retinene.

4. All properties of the squid visual purple thus far investigated closely resemble those of the vertebrate visual purples, with the exception that

the squid pigment is relatively unstable thermally and is not bleached by light.

5. It is suggested that the light sensitivity of the normal squid photopigment may be independent of its light stability.

BIBLIOGRAPHY

1. Krohn, A., *Nov. act. acad. Caesar. Leopoldino-Carolinae nat. cur.*, **19**, pt. 2, 43 (1842).
2. Lythgoe, R. J., and Quilliam, J. P., *J. Physiol.*, **94**, 399 (1938).
3. Lythgoe, R. J., *J. Physiol.*, **89**, 331 (1937).
4. Krause, A., *Am. J. Physiol.*, **140**, 40 (1943).
5. Krause, A., *Am. J. Physiol.*, **145**, 561 (1946).
6. Bliss, A. F., *J. Biol. Chem.*, **172**, 165 (1948).
7. Wald, G., *J. Gen. Physiol.*, **19**, 351 (1935).
8. Ball, S., Collins, F. D., Morton, R., and Stubbs, A. L., *Nature*, **161**, 424 (1948).
9. Wald, G., *Am. J. Physiol.*, **133**, P 479 (1941).
10. Bliss, A. F., *J. Gen. Physiol.*, **26**, 361 (1943).
11. White, G., *Biol. Bull.*, **47**, 265 (1924).
12. Hartline, H. K., Milne, L. J., and Wagman, I. H., *Federation Proc.*, **6**, 124 (1947).
13. Hecht, S., Shlaer, S., and Pirenne, M., *J. Gen. Physiol.*, **25**, 819 (1942).
14. Hartline, H. K., and McDonald, P., *J. Cell. and Comp. Physiol.*, **30**, 225 (1947).
15. Saito, Z., *Tohoku J. Exp. Med.*, **32**, 432 (1938).
16. Bliss, A. F., *J. Gen. Physiol.*, **29**, 277 (1946).
17. Escher-Desrivières, J., Lederer, E., and Verrier, M.-L., *Compt. rend. Acad.*, **207**, 1447 (1938).
18. Bliss, A. F., *Biol. Bull.*, **93**, in press.

STUDIES ON FREE ERYTHROCYTE PROTOPORPHYRIN, PLASMA COPPER, AND PLASMA IRON IN PROTEIN-DEFICIENT AND IRON-DEFICIENT SWINE*

By GEORGE E. CARTWRIGHT AND MAXWELL M. WINTROBE

(From the Department of Medicine, University of Utah School of Medicine, Salt Lake City)

(Received for publication, July 1, 1948)

In a previous communication (1) values for free erythrocyte protoporphyrin, plasma copper, and plasma iron in normal and in pyridoxine-deficient swine were presented. The values in the normal group were found to be 118 ± 43.4, 206 ± 26.3, and 169 ± 38.8 γ per cent respectively. In the pyridoxine-deficient group on the other hand, the values were 47 ± 13.6, 160 ± 38.8, and 468 ± 166.6 γ per cent, respectively. Because of the small amount of free protoporphyrin in the erythrocytes of the pyridoxine-deficient animals, and because a fall in the erythrocyte protoporphyrin was noted long before the development of anemia in animals in which pyridoxine deficiency was induced, it was suggested that the fundamental disturbance in anemia due to pyridoxine deficiency may be a failure in protoporphyrin synthesis.

The purpose of this report is to present the results of similar determinations in swine with anemia due to protein deficiency, iron deficiency, and acute hemorrhage. In addition, values for the iron-binding capacity of the serum in normal, protein-deficient, and iron-deficient swine are given.

Materials and Methods

For this study thirty-nine weanling pigs were used. Twenty-four animals were placed on a low protein diet and fifteen were fed a diet low in iron but adequate in all other respects.

The basal diet of the low protein group consisted of either Sheffield "new process" casein 10.0 per cent, sucrose 73.8 per cent, lard 11.0 per cent, and swine Salt Mixture 3 (2) 5.2 per cent, or casein 10.0 per cent, sucrose 57.7 per cent, lard 27.1 per cent, salt mixture 5.2 per cent. The basal diet of the iron-deficient group consisted of casein 26.1 per cent, sucrose 57.7 per cent, lard 11.0 per cent, and swine Salt Mixture 3 (with iron pyrophosphate omitted) 5.2 per cent. In addition, all animals were given cod liver oil (Mead Johnson, 1800 units of vitamin A, 175 units of vitamin D per gm.) 0.5 gm. per kilo of body weight daily, or Natola (Parke, Davis, 55,000

---

* Aided by a grant from the United States Public Health Service, and by grants from The Upjohn Company and Parke, Davis and Company.

units of vitamin A, 11,000 units of vitamin D per gm.) 0.056 gm. per kilo of body weight per week. Vitamins were supplied in crystalline form in capsules and were administered orally three times a week. The quantities of crystalline vitamins were, with the exceptions noted below, thiamine hydrochloride 0.25, riboflavin 0.12, nicotinic acid 1.20, pyridoxine hydrochloride 0.20, calcium pantothenate 0.50, choline chloride 10.0, *p*-aminobenzoic acid 0.10, inositol 0.10. All animals except Pigs 9-13 to 9-63, inclusive, received crystalline biotin 50 γ per kilo of body weight per week intramuscularly. Pigs 10-45 to 10-59, inclusive, received the high fat diet and 50.0 mg. of choline rather than 10.0 mg. Pigs 10-24 to 10-44, inclusive, were given no *p*-aminobenzoic acid or inositol. Pteroylglutamic acid, either 30 or 100 mg. per kilo of body weight daily, was added to the vitamin supplement of Pigs 10-24 to 10-58. Pigs 10-37, 10-40, 10-41, 10-42, 10-43, and 10-44 received no niacin in the vitamin supplements during the first part of the experiment but had been treated with adequate amounts of this vitamin for at least 30 days prior to the determinations included in the present report. Full details of the experimental methods have been published elsewhere (2).

Erythrocyte protoporphyrin determinations were made by the method of Grinstein and Watson (3). Plasma copper was measured by the method of Cartwright, Jones, and Wintrobe (4). For the determination of plasma iron the method of Kitzes, Elvehjem, and Schuette (5) as well as the method of Barkan and Walker (6) was used. A modification[1] of the method of Schade and Caroline (7) was used for the measurement of the iron-binding capacity of serum. Serum proteins were determined by the biuret method (8) with the modification of Kingsley (9). By this procedure the values for ten control animals (26 per cent casein) were found to be as follows: total serum protein 6.15 ± 0.437 gm. per cent, serum albumin 3.72 ± 0.173 gm. per cent, serum globulin 2.43 ± 0.223 gm. per cent.

Results

Protein Deficiency—The morphologic characteristics of the anemia observed in these animals are presented in Table I. The anemia was usually mild in degree. The mean value for the volume of packed red cells for twenty-two pigs was 35.7 ml. per 100 ml. The mean value for normal pigs is approximately 45.0 ml. per 100 ml. and values below 40 ml. are rarely encountered. The anemia was normocytic and normochromic and was not accompanied by reticulocytosis. Leucopenia and thrombocytopenia were not present.

Chemical findings are presented in Table II. In all the animals hypoproteinemia and especially hypoalbuminemia were present. The mean value for erythrocyte protoporphyrin was not significantly altered from the normal, being 124 ± 35.6 γ per 100 ml. of red blood cells. The mean value for plasma iron was 115 ± 30.1 γ per cent as compared with 169 ± 38.8 for the normal group. This represents a significant lowering of plasma iron, although not to the extent seen in iron-deficient animals. In no in-

---

[1] Cartwright, G. E., Black, P., and Wintrobe, M. M., *J. Clin. Invest.*, in press.

TABLE I

Morphologic Data for Protein-Deficient Swine

Pig No.	Age at beginning of experiment	Days on experiment	Weight	Red blood cells	Hb	Volume of packed red blood cells	Mean corpuscular volume	Mean corpuscular Hb	Mean corpuscular Hb concentration	Reticulocytes	White blood cells	Platelets
	days		*kg.*	*millions per c.mm.*	*gm. per cent*	*ml. per 100 ml.*	*cu. micra*	*micro-micro-grams*	*per cent*	*per cent*	*thousands per c.mm.*	*thousands per c.mm.*
9-61	31	134	22.7	5.75	10.4	31.5	55	18	33	0.4	12.6	566
9-62	21	154	18.6	5.72	11.5	34.5	60	20	33	2.8	19.3	570
9-63	21	112	6.4	4.13	6.5	21.0	51	16	31	2.8	6.2	354
10-24	70	183	49.8	7.40	12.7	39.0	53	17	33	0.4	17.7	400
10-25	60	183	54.5	7.83	13.8	41.0	52	18	34	0.8	27.4	490
10-27	70	183	44.0	6.94	12.7	39.0	56	18	33	1.5	17.5	300
10-29	60	183	65.6	6.75	13.8	39.0	58	20	35	0.6	17.0	430
10-33	70	183	19.5	7.21	12.1	38.0	53	17	32	4.8	21.8	400
10-34	60	183	79.7	7.35	12.9	41.0	54	17	31	0.5	24.1	380
10-35	70	183	56.4	7.01	11.9	35.2	50	17	34	0.9	15.4	380
10-37	21	206	31.7	6.22	11.7	36.0	58	19	32	2.2	13.9	430
10-40	21	206	17.0	6.30	10.9	35.0	55	17	31	1.4	13.6	350
10-41	21	206	39.1	7.10	13.8	40.6	57	19	34	1.8	11.5	330
10-42*	21	206	15.9	2.29	3.2	12.0	52	14	28	9.8	18.0	280
10-43†	21	180	21.6	4.27	7.8	24.0	56	18	32	0.2	17.7	380
10-44	21	206	30.7	6.33	11.7	37.0	59	19	32	3.0	20.4	340
10-45	27	156	13.7	6.96	12.0	36.0	52	17	33	1.0	15.1	440
10-46	27	156	17.7	6.36	11.8	35.2	55	19	33	2.6	17.8	480
10-47	27	156	20.5	5.31	8.9	27.0	51	16	33	2.0	13.7	490
10-48	27	156	10.7	6.35	12.0	36.4	57	19	33	1.0	14.5	460
10-50	27	156	19.3	6.51	11.3	34.4	53	17	33	0.6	12.3	410
10-57	30	137	20.4	5.87	12.3	37.2	63	21	33	0.4	10.9	400
10-58	30	137	22.5	6.19	12.1	35.6	58	20	34	0.4	14.0	430
10-59	30	137	17.6	6.45	11.7	35.4	55	18	33	1.2	17.2	450
Mean..	38	168	30.8	6.46	11.8	35.7	55	18	33	1.5	16.1	422

* Terminal hemorrhage; values not included in the mean.

† Infection; values not included in the mean.

stance were values as high as those seen in normal animals observed in protein-deficient pigs. The correlation between the degree of hypoproteinemia and the iron-binding capacity of the serum is shown in Fig. 1. With a reduction in the total serum proteins there was a proportional reduction in the iron-binding capacity. It is of interest that even though the iron-binding protein has been shown to be a globulin, the decrease in iron-binding capacity was correlated with the decrease in serum albumin rather than globulin. This may be explained by the fact that the iron-binding protein is a globulin of low molecular weight (90,000). It follows in great

TABLE II

Chemical Data for Protein-Deficient Swine

Pig No.	Erythrocyte protoporphyrin	Plasma copper	Plasma iron	Total iron-binding capacity of serum	Total serum proteins	Serum albumin	Serum globulin
	γ per 100 ml. red blood cells	*γ per cent*	*γ per cent*	*γ per cent*	*gm. per cent*	*gm. per cent*	*gm. per cent*
9-61	196	129	166		4.43	1.76	2.67
9-62	100	131	123		4.49	1.84	2.65
9-63	212	87	95		3.61	0.83	2.78
10-24	110	157	177	227	4.55	1.32	3.23
10-25	79	175	101	166	5.60	1.35	4.25
10-27	113	131	168	283	4.48	1.29	3.19
10-29	80	144	103	303	5.15	2.30	2.85
10-33	87	115	110	160	3.55	0.68	2.87
10-34	137	197	60	205	5.95	1.24	4.71
10-35	100	137	116	266	3.81	1.02	2.79
10-37	154	129	108	308	5.04	1.39	3.65
10-40	156	87	103	208	4.31	0.91	3.40
10-41	134	122	123	278	4.35	1.38	2.97
10-42*	224	113	19	269	3.24	0.93	2.31
10-43†	183	124	34	184	3.16	0.66	2.50
10-44	109	137	131	306	4.22	1.53	2.69
10-45	129	127	108	258	4.38	1.31	3.07
10-46	111	157	118	413	4.75	1.97	2.78
10-47	111	179	140	360	4.92	1.94	2.98
10-48	109	137	66	261	4.51	1.61	2.90
10-50	78	131	72	252	4.51	1.58	2.93
10-57	162	142	131	481	5.49	2.91	2.58
10-58	123	142	106	424	5.02	2.72	2.30
10-59	144	169	105	505	5.02	2.38	2.64
Mean.......	124	139	115	298	4.64	1.60	3.04

* Terminal hemorrhage; values not included in mean.

† Infection; values not included in mean.

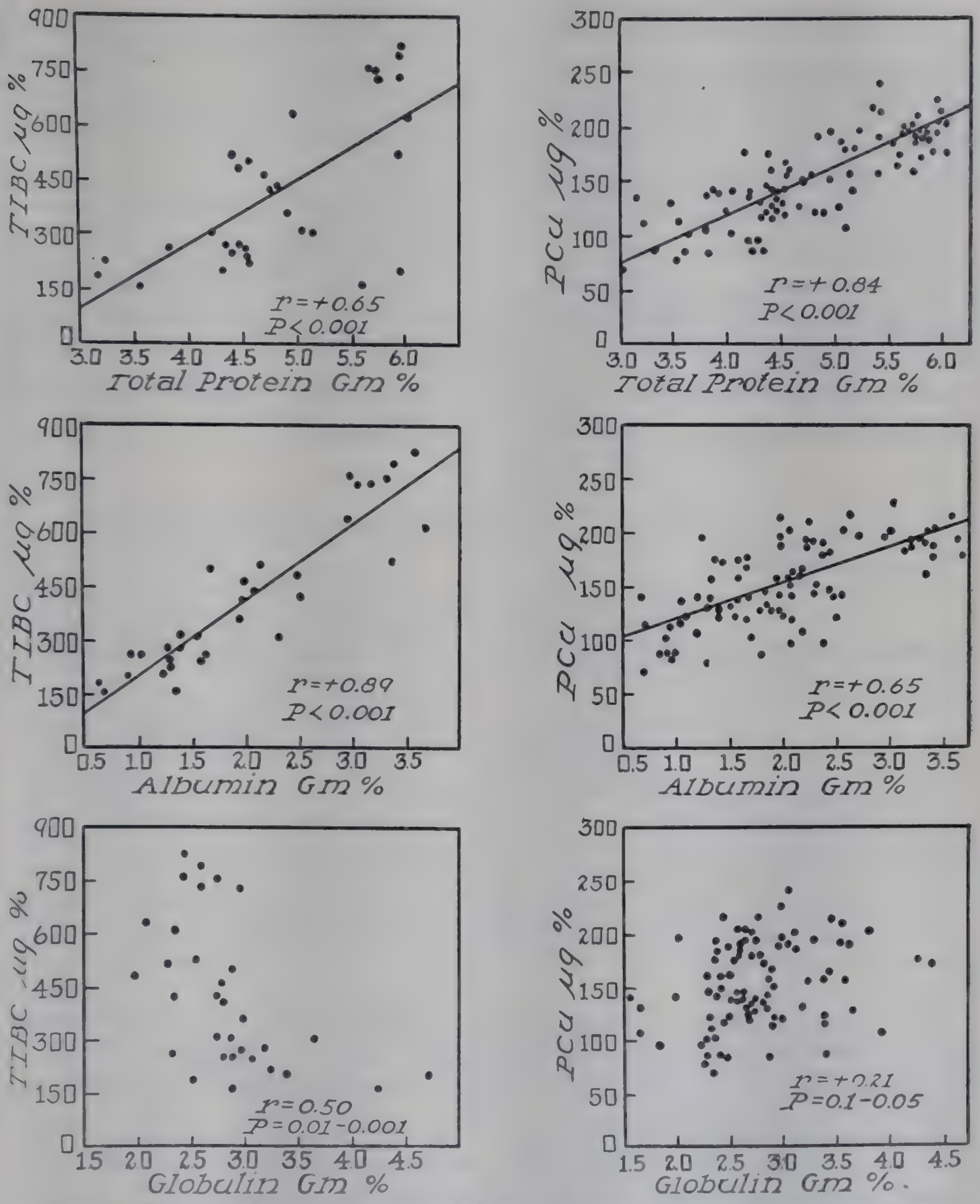


FIG. 1. The correlation between total serum protein, serum albumin, and serum globulin with the iron-binding capacity of the serum and the plasma copper. *r* refers to the correlation coefficient. *P* refers to the probability that a correlation as large as that indicated could occur by chance in a population in which no correlation exists. A value of *P* less than 0.05 is considered significant.

part the albumin fraction in the usual 23 per cent sodium sulfate fractionation procedure.

Hypocupremia was observed consistently in the hypoproteinemic animals. The mean value was 139 ± 26.4 γ per cent as compared with a mean value of 206 ± 26.3 γ per cent in the control group. The correlation between the plasma copper and total serum protein, albumin, and globulin is shown in Fig. 1. The degree of hypocupremia appears to be correlated closely with the degree of hypoalbuminemia.

It is of interest that in the two pigs (Nos. 10-42, 10-43) with complicating disorders there was an increase in erythrocyte protoporphyrin and a decrease in plasma iron (Table II). In one (Pig 10-42) there was a severe terminal gastric hemorrhage and in the other (Pig 10-43) a terminal infection accompanied by leucocytosis developed. In human subjects both hemorrhage and infection are associated with a rise in erythrocyte protoporphyrin and a decrease in plasma iron (10).

TABLE III

Effects of Protein Therapy on Blood of Three Protein-Deficient Animals

Days of treatment 53.

Pig No.	Condition	Volume of packed red blood cells	Erythrocyte protoporphyrin	Plasma copper	Plasma iron	Total iron-binding capacity of serum	Total serum proteins	Serum albumin	Serum globulin
		ml. per 100 ml.	*γ per 100 ml. red blood cells*	*γ per cent*	*γ per cent*	*γ per cent*	*gm. per cent*	*gm. per cent*	*gm. per cent*
10-57	Deficient	33.8	123	127	123	480	4.48	2.49	1.99
	Treated	43.0	114	218	131	790	6.34	3.61	2.73
10-58	Deficient	35.0	123	124	99	425	4.42	2.06	2.36
	Treated	44.0	100	201	39	740	5.96	3.01	2.95
10-59	Deficient	30.0	115	161	105	500	4.55	1.67	2.88
	Treated	40.0	92	205	71	800	6.02	3.38	2.64

The effects of protein therapy in three protein-deficient animals are shown in Table III and the effects in a single animal are presented in detail in Fig. 2. An increase in the casein content of the diet from 10 to 26 per cent produced a mild reticulocytosis, and was followed by an increase in the volume of packed red cells, plasma copper, iron-binding capacity, and serum proteins to normal. There was no significant change in the erythrocyte protoporphyrin. One animal (Pig 10-58, Fig. 2) developed persistent hypoferremia, probably because of the increased erythropoiesis, but in the other two the hypoferremia was only transient and was observed during the period of rapid blood regeneration.

Iron Deficiency—Morphologic and chemical findings in the pigs made iron-deficient are presented in Table IV. The anemia was severe in degree, microcytic and hypochromic, and was accompanied by reticulocytosis. Significant alterations from the normal erythrocyte protoporphyrin and

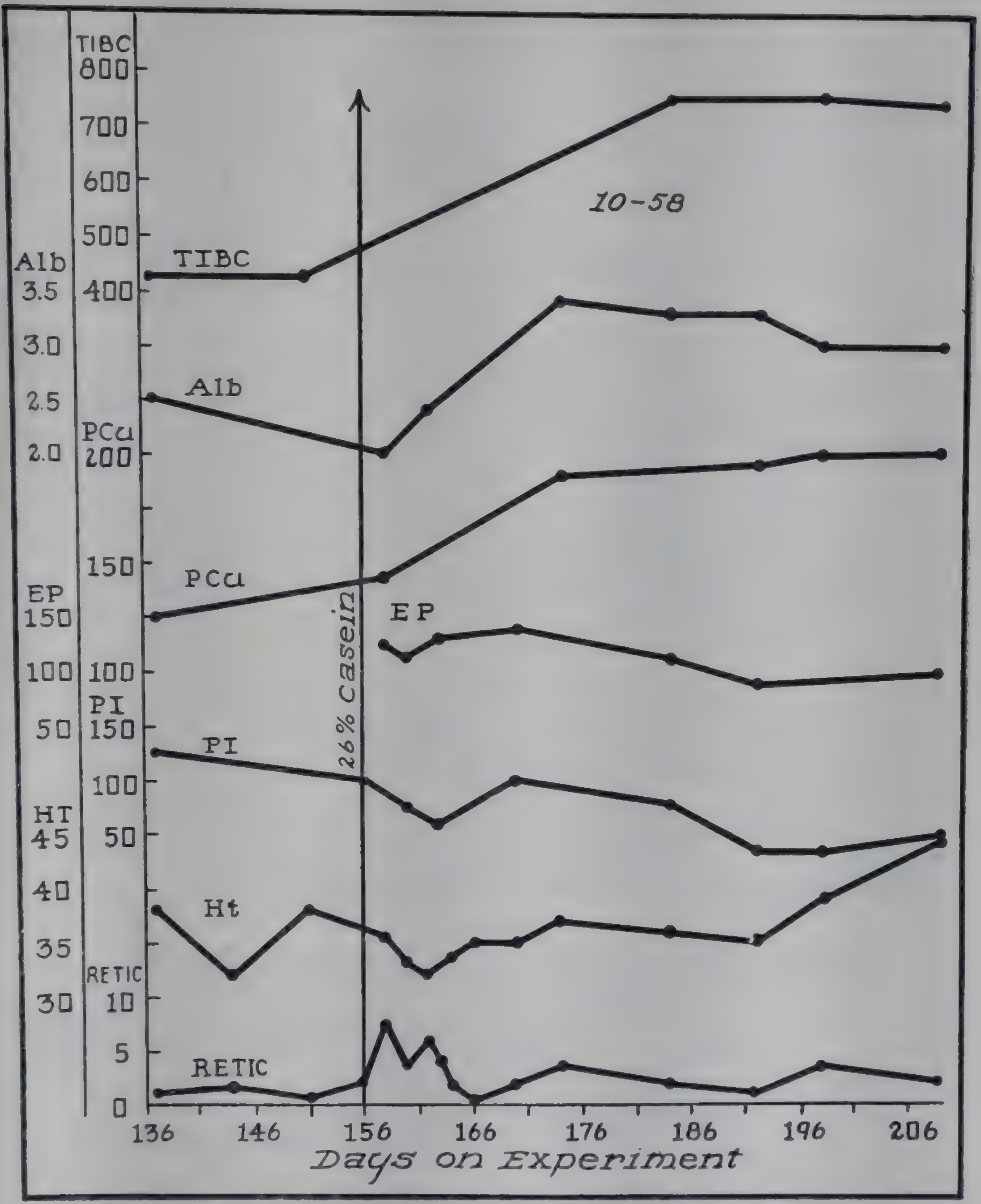


FIG. 2. The effect of protein therapy in a pig (No. 10-58) deficient in protein. The casein in the diet was increased from 10 to 26 per cent on the 156th day. *TIBC* represents total iron-binding capacity of the serum in γ per cent; *Alb* serum albumin in gm. per cent; *PCu* plasma copper in γ per cent; *EP* free erythrocyte protoporphyrin in γ per 100 ml. of red cells; *PI* plasma iron in γ per cent; *Ht* volume of packed red cells in ml. per 100 ml.; *Retic* reticulocytes in per cent.

plasma copper were not noted. The mean value for erythrocyte protoporphyrin was 127 ± 31.4 γ per 100 ml. of red blood cells and for plasma copper 207 ± 23.8 γ per cent. The anemia was, however, associated with

TABLE IV

Morphologic and Chemical Changes in Blood of Swine Fed Iron-Deficient Diets

Pig No.	Age at beginning of experiment	Days on experiment	Weight	Red blood cells	Hb	Volume of packed red blood cells	Mean corpuscular volume	Mean corpuscular Hb	Mean corpuscular Hb concentration	Reticulocytes	Erythrocyte protoporphyrin	Plasma copper	Plasma iron
	days		*kg.*	*million per mm.*	*gm. per cent*	*ml. per 100 ml.*	*cu. micra*	*micro-micro-grams*	*per cent*	*per cent*	*γ per 100 ml. red blood cells*	*γ per cent*	*γ per cent*
9-13	35	175	75.5	7.23	7.0	27.0	38	9	26	5.8	95	216	32
9-14	35	160	59.2	6.27	6.9	25.5	41	11	27	6.3	140	257	38
9-24	21	152	25.6	6.13	3.7	18.5	30	6	20	4.4	90	201	17
9-25	21	163	53.6	6.29	5.2	21.5	34	8	24	6.2	125	199	24
9-26	21	194	54.5	4.26	3.7	15.0	35	9	25	6.6	105	206	40
9-27	21	194	65.4	5.36	5.1	19.5	36	10	26	1.2	86	189	41
9-46	21	63	25.9	4.30	3.9	15.5	36	9	25	4.2	174	198	26
9-47	21	64	27.0	5.94	5.5	22.0	37	9	25	2.8	116	193	24
9-48	21	78	26.3	4.05	3.0	12.5	31	7	24	5.4	177	187	29
9-49	21	78	23.4	3.78	2.4	10.0	26	6	24	4.6	178	177	29
9-50	21	78	30.6	4.60	4.0	16.5	36	9	24	7.2	116	177	32
9-51	21	78	48.8	5.43	5.4	20.0	37	10	27	5.8	141	221	26
10-16*	144	97	70.4	4.50	4.8	17.4	38	11	28	13.4	101	253	39
10-17*	144	97	75.2	6.20	5.2	21.0	34	8	25	18.4	113	209	30
10-18*	144	97	82.2	5.83	5.4	21.0	36	9	26	18.2	148	220	40
Mean..	47	118	49.5	5.34	4.7	18.8	35	9	25	7.4	127	207	31

* Hemorrhage superimposed on dietary deficiency of iron.

TABLE V

Effects of Acute and Chronic Hemorrhage on Blood of Three Pigs

Pig No.	Period	Volume of packed red blood cells	Erythrocyte protoporphyrin	Plasma copper	Plasma iron	Total iron-binding capacity of serum
		ml. per 100 ml.	*γ per 100 ml. red blood cells*	*γ per cent*	*γ per cent*	*γ per cent*
10-16	Control	42.2	105	195	152	
	Acute hemorrhage	17.2	180	264	16	
	Iron deficiency	17.4	101	253	39	340
	Treated ($FeSO_4$)	50.0	132	207	177	667
10-17	Control	40.0	120	209	105	
	Acute hemorrhage	20.0	239	293	27	
	Iron deficiency	21.0	113	209	30	392
	Treated ($FeSO_4$)	48.0	97	214	121	588
10-18	Control	37.6	124	197	200	
	Acute hemorrhage	20.8	239	286	29	
	Iron deficiency	21.0	148	220	40	890
	Treated ($FeSO_4$)	45.8	100	205	172	572

a severe hypoferremia, the mean plasma iron value being 31 ± 7.2 γ per cent.

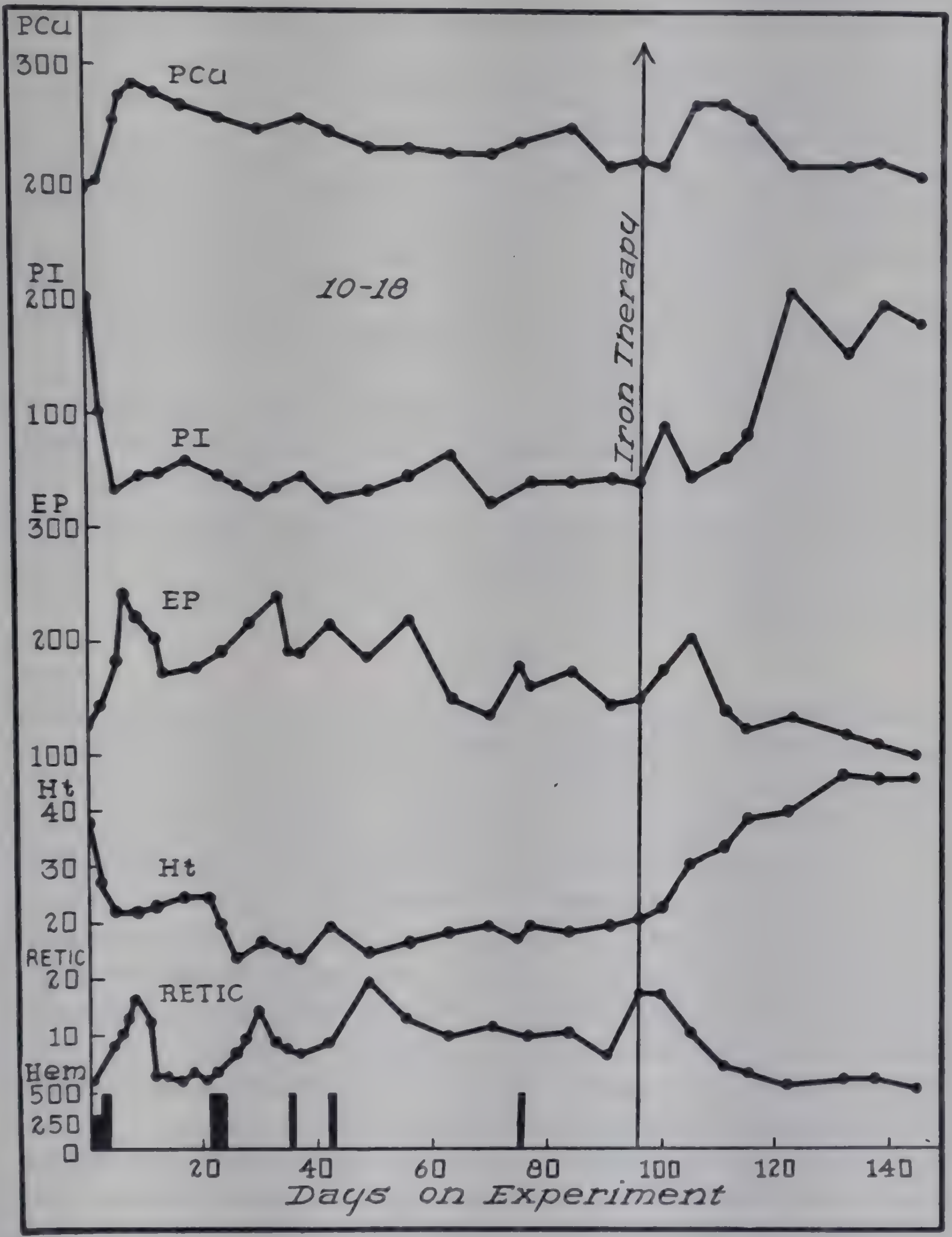


FIG. 3. The effect of phlebotomy and a diet low in iron on the blood of a pig (No. 10-18). Under *Hem* the quantity of blood removed is expressed in ml. On the 96th day of the experiment the pig was treated with 2 gm. of ferrous sulfate daily. The legends are the same as in Fig. 2.

Three animals (Pigs 10-16, 10-17, 10-18, Table V) were fed a diet low in iron and 1500 ml. of blood were removed in 4 days. This resulted in all three animals in severe normocytic normochromic anemia, reticulocytosis, severe hypoferremia, and a significant although not marked rise in erythrocyte protoporphyrin (219 ± 33.8 γ per 100 ml. of red cells) and plasma copper (281 ± 15.1 γ per cent). The effects of venesection are shown in detail for one animal (Pig 10-18) in Fig. 3. The low iron diet and phlebotomy were then continued until the animals developed severe microcytic hypochromic anemia. At this time the values for erythrocyte protoporphyrin and plasma copper were normal, although the hypoferremia persisted (Table V and Fig. 3). These animals were then treated with large doses of ferrous sulfate (2 gm. per pig per day) orally. In each animal there was a transient increase in erythrocyte protoporphyrin and plasma copper, followed by a decrease in reticulocytes and a return of all of the constituents studied to normal.

Determinations of the iron-binding capacity of the serum were made in only three iron-deficient pigs (Table V). In two of the animals the iron-binding capacity was reduced and in the third no change from the normal was noted. More data are needed before definite conclusions can be drawn, especially since the results are not consistent and are not in accord with those found in human subjects deficient in iron.[1]

DISCUSSION

Protein deficiency anemia was found to be accompanied by a severe hypoalbuminemia and a normal amount of free protoporphyrin in the erythrocytes. In view of the fact that protoporphyrin is believed to be synthesized from certain amino acid precursors (11) this finding is of some interest and would seem to indicate that porphyrin synthesis has priority over growth and the maintenance of normal serum proteins. This is in accord with the conclusion of Whipple and his group (12) that the body gives preference to hemoglobin production as compared with serum protein production. That severely hypoproteinemic animals can synthesize protoporphyrin readily is indicated by the fact that a substantial rise in erythrocyte protoporphyrin occurred in Pig 10-42 following a severe hemorrhage. Pig 10-43 responded in a similar fashion to an infection.

The slight reduction in plasma iron in the protein-deficient animals in comparison with normal control animals may be explained by the fact that the amount of iron carried in the serum is limited, at least under normal circumstances, by the amount of iron-binding protein in the blood (β_1-globulin, Fraction IV-7 of Cohn[1]). In the protein-deficient animals this protein appeared to be markedly reduced, since the mean iron-binding capacity was 298 ± 98.3 γ per cent as compared with 845 ± 90.6 γ per cent in ten control pigs. The mean per cent saturation of the protein with iron ((plasma iron)/(total iron-binding capacity of the serum)) for the deficient animals was 38.6 and for the control animals 31.0. Thus it would seem that in both situations the iron-binding protein was maintained at about one-third saturation.

The moderate hypocupremia observed in the animals deficient in protein may indicate that the copper in the plasma is bound by a protein and that in the deficient animals this protein was markedly reduced. Since the degree of hypocupremia appeared to be correlated closely with the degree of hypoalbuminemia, this may indicate that the copper is bound to albumin or to globulin of low molecular weight. Cohn and his group have presented evidence that the β_1-globulin Fraction IV-7 may bind copper as well as iron reversibly although preference is given to iron.[2]

The fact that in protein deficiency there is no abnormality in erythrocyte protoporphyrin, only a slight reduction in plasma iron, and a significant reduction in plasma copper contrasts with the findings in the anemia of infection which is characterized by increased erythrocyte protoporphyrin, hypoferremia, and hypercupremia. It was suggested elsewhere (13) that the anemia of infection may be related to the disturbance in protein metabolism which accompanies trauma and various other types of tissue injury. The present observations indicate that the anemia of infection is not due simply to protein deficiency resulting from increased nitrogen excretion in the urine.

The normal erythrocyte protoporphyrin and plasma copper values in the iron-deficient pigs do not correspond with findings in patients with iron deficiency. Studies in this laboratory (10) have indicated that in human subjects microcytic hypochromic anemia due to a deficiency of iron is accompanied by a marked increase in erythrocyte protoporphyrin and plasma copper. The reason for this difference is not obvious. Two explanations can be suggested. Since the normal values for erythrocyte protoporphyrin and plasma copper in swine and man are quite different (1, 10) the failure of these to increase significantly in iron deficiency in swine may represent a species difference. Another possibility is that the high erythrocyte protoporphyrin values and hypercupremia which accompany microcytic hypochromic anemia in man are not due to iron deficiency *per se* but are due to some other cause. This is suggested by the fact that acute hemorrhage in both swine and man is accompanied by an increase in free erythrocyte protoporphyrin and plasma copper. Further work in various species under differing experimental conditions is needed before a definite conclusion can be drawn.

The results of the studies on erythrocyte protoporphyrin, plasma copper, and plasma iron in normal swine and in swine with anemia due to pyridoxine deficiency, protein deficiency, iron deficiency, and acute hemorrhage are summarized in Table VI. In general it may be concluded that pyridoxine deficiency is characterized by a microcytic normochromic anemia with a marked decrease in erythrocyte protoporphyrin, a slight reduction in plasma copper, and a marked increase in plasma iron. Chronic protein deficiency is characterized by a normocytic normochromic anemia, normal erythrocyte protoporphyrin, a moderate hypocupremia, and a slight reduction in plasma iron. Chronic iron deficiency is characterized by a microcytic hypochromic anemia, hypoferremia, and normal erythrocyte protoporphyrin and plasma copper. Acute hemorrhage results in a normocytic normochromic anemia, an increase in erythrocyte protoporphyrin, and hypercupremia and hypoferremia.

---

[2] Cohn, E. J., personal communication.

TABLE VI

Summary of Data

Group	Type of anemia	Erythrocyte protoporphyrin	Plasma copper	Plasma iron	Total iron-binding capacity of serum
		γ per 100 ml. red blood cells	*γ per cent*	*γ per cent*	*γ per cent*
Normal	Normocytic, normochromic	118 ± 43.4	206 ± 26.3	169 ± 38.8	845 ± 90.6
Pyridoxine-deficient	Microcytic, normochromic	47 ± 13.6	160 ± 38.8	468 ± 166.6	
Protein-deficient	Normocytic, normochromic	124 ± 35.6	139 ± 26.4	115 ± 30.1	298 ± 98.3
Iron-deficient	Microcytic, hypochromic	127 ± 31.4	207 ± 23.8	31 ± 7.2	
Acute hemorrhage	Normocytic, normochromic	219 ± 33.8	281 ± 15.1	24 ± 7.0	

SUMMARY

1. Chronic protein deficiency in swine resulted in a mild normocytic normochromic anemia which was accompanied by normal erythrocyte protoporphyrin values (124 ± 35.6 γ per 100 ml. of red blood cells), slightly reduced plasma iron values (115 ± 30.1 γ per cent), a moderate hypocupremia (139 ± 26.4 γ per cent), and a marked reduction in the iron-binding capacity of the serum (298 ± 98.3 γ per cent).

2. Chronic iron deficiency in swine resulted in a severe microcytic hypochromic anemia which was accompanied by reticulocytosis, normal values for erythrocyte protoporphyrin (127 ± 31.4 γ per 100 ml. of red blood cells), normal plasma copper values (207 ± 23.8 γ per cent), and marked hypoferremia (31 ± 7.2 γ per cent).

3. Acute hemorrhage in swine resulted in a severe normocytic normo-

chromic anemia which was accompanied by reticulocytosis, an increase in erythrocyte protoporphyrin (219 ± 33.8 γ per 100 ml. of red cells), an elevation in plasma copper (281 ± 15.1 γ per cent), and marked hypoferremia (24 ± 7.0 γ per cent).

4. The results of morphologic and chemical studies of the blood of normal swine and of swine with anemia due to pyridoxine deficiency, protein deficiency, iron deficiency, and acute hemorrhage are summarized.

The vitamins, with the exception of pteroylglutamic acid and biotin, were kindly furnished by Merck and Company, Rahway, New Jersey, through the courtesy of the late Dr. D. F. Robertson. The pteroylglutamic acid was kindly furnished by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, through the courtesy of Dr. T. H. Jukes and Dr. S. M. Hardy. Biotin was obtained from Hoffmann-La Roche, Inc., Nutley, New Jersey, through the courtesy of Dr. E. L. Sevringhaus. Natola was supplied by Parke, Davis and Company, Detroit, Michigan, through the courtesy of Dr. E. A. Sharp. Cod liver oil was supplied by Mead Johnson and Company, Evansville, Indiana, through the courtesy of Dr. W. M. Cox, Jr.

We are indebted to Miss Helen Ashenbrucker, Miss Pauline Black, Miss Mary Iles, Miss Betty Tatting, Mrs. Darlene Kehl, Mr. George Trappett, and Mr. Ocie Hadley for technical assistance.

BIBLIOGRAPHY

1. Cartwright, G. E., and Wintrobe, M. M., *J. Biol. Chem.*, **172**, 557 (1948).
2. Wintrobe, M. M., Miller, J. L., and Lisco, H., *Bull. Johns Hopkins Hosp.*, **67**, 377 (1940).
3. Grinstein, M., and Watson, C. J., *J. Biol. Chem.*, **147**, 675 (1943).
4. Cartwright, G. E., Jones, P. J., and Wintrobe, M. M., *J. Biol. Chem.*, **160**, 593 (1945).
5. Kitzes, G., Elvehjem, C. A., and Schuette, H. A., *J. Biol. Chem.*, **155**, 653 (1944).
6. Barkan, G., and Walker, B. S., *J. Biol. Chem.*, **135**, 37 (1940).
7. Schade, A. L., and Caroline, L., *Science*, **104**, 340 (1946).
8. Weichselbaum, T. E., *Am. J. Clin. Path.*, **16**, *Tech. Sect.*, **10**, 40 (1946).
9. Kingsley, G. R., *J. Biol. Chem.*, **133**, 731 (1940).
10. Cartwright, G. E., Huguley, C. M., Ashenbrucker, H., Fay, J. M., and Wintrobe, M. M., *Blood*, **3**, 501 (1948).
11. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **166**, 621 (1946).
12. Robscheit-Robbins, F. S., Madden, S. C., Rowe, A. P., Turner, A. P., and Whipple, G. H., *J. Exp. Med.*, **72**, 479 (1940).
13. Greenberg, G. R., Ashenbrucker, H., Lauritsen, M., Worth, W., Humphreys, S. R., and Wintrobe, M. M., *J. Clin. Invest.*, **26**, 121 (1947).

ELECTROPHORETIC ANALYSES OF SERA OF NORMAL AND HYPOPROTEINEMIC SWINE*

By GEORGE E. CARTWRIGHT, EMIL L. SMITH, DOUGLAS M. BROWN, AND MAXWELL M. WINTROBE

(From the Department of Medicine, and the Laboratory for the Study of Hereditary and Metabolic Disorders, University of Utah School of Medicine, Salt Lake City)

(Received for publication, July 1, 1948)

In the preceding paper (1) studies on free erythrocyte protoporphyrin, plasma copper, and plasma iron in protein-deficient and iron-deficient swine are reported. These experiments made animals available for electrophoretic studies which were severely hypoproteinemic as a result of a prolonged dietary restriction of protein. Studies on the sera of a number of these animals are presented in this paper.

Electrophoretic analyses of the sera of normal swine have been reported previously by Svensson (2), Moore (3), Deutsch and Goodloe (4), and Koenig and Hogness (5). No studies have been reported on the serum proteins of hypoproteinemic swine. However, Zeldis *et al.* (6) studied the electrophoretic patterns of the plasma of dogs following long and continued restriction of dietary protein. They found a marked decrease in albumin levels and essentially no change in the plasma globulin concentrations. The degree of depletion of "electrophoretic" albumin was considerably greater than that of "chemical" albumin. When large amounts of protein were fed to such dogs, complete restoration of the normal plasma albumin took place in several weeks. They concluded that "plasma globulins, in contrast to plasma albumin, enjoy prior demands on the total available pool of body protein materials under emergency conditions." They noted during depletion an increase in the total electrophoretic globulin, especially in the α-globulin areas, which was attributed to an increase in plasma lipides. Chow (7) in similar studies on dogs during chronic depletion by both plasmaphoresis and protein-free feeding observed similar changes. Studies in human subjects, hypoproteinemic as a result of malnutrition, have revealed a drop in the albumin content of the serum associated with a corresponding increase in α-globulins (7, 8).

Observations

The basal diet and vitamin supplements of the animals used in this study have been described in detail in the preceding publication (1). Three of

---

* This investigation was aided by a grant from the United States Public Health Service, and by grants from The Upjohn Company and Parke, Davis and Company.

the control animals (Pigs 10-57, 10-58, 10-59) were maintained on a low protein (10 per cent casein) diet for 156 days. At this time the casein in the diet was increased to 26 per cent. The electrophoretic analyses were made on the 211th day of the experiment, 55 days after the protein had been increased to 26 per cent. The two other controls (Pigs 10-16, 10-17) were maintained on a diet containing 26 per cent casein from the beginning of the experiment. The electrophoretic analyses were made on the 240th

TABLE I

Electrophoretic Analyses of Sera of Normal and Protein-Deficient Swine

The results are given as percentage of the total refractive increment. The numbers in parentheses refer to mobility × 10^{-5} sq. cm. per volt per second and are negative in sign.

Pig No.	Protein N	γ_2	γ_1	β_2	β_1	$\alpha_2 + \alpha_3$	α_1	Albumin
Controls								
	mg. per ml.							
10-57	8.75	12 (1.7)	9 (2.8)	5 (3.2)	5 (3.7)	16 (4.4)	4 (5.5)	49 (6.9)
10-58	8.27	9 (1.9)	13 (2.8)	5 (3.4)	2 (3.8)	19 (4.7, 4.2)	4 (5.7)	48 (7.1)
10-59	11.33	13 (1.6)	12 (2.5)	5 (3.1)	3 (3.4)	20 (4.1)	3 (5.1)	44 (6.1)
10-16	8.26		13* (1.6)		16* (2.8)	23 (4.3)	6 (5.5)	42 (6.4)
10-17	13.37	21 (1.8)	7 (2.7)	4† (2.9)	4 (3.4)	20 (4.2)	4 (5.2)	40 (6.2)
Protein-deficient								
10-40	4.89	28 (1.9)	6 (2.7)	3 (3.0)	6 (3.3)	39 (4.0)	10 (5.5)	8 (6.5)
10-41	7.45		23* (2.0)	6 (3.1)	7 (3.5)	38 (4.6)	8 (5.7)	18 (6.8)
10-44	7.61	20 (1.9)	12 (2.7)		3 (3.3)	39 (4.0)	9 (5.4)	17 (6.2)
10-45	5.59		23* (1.4)	9 (2.7)	8 (3.4)	36 (5.1, 4.4)	6 (5.8)	18 (6.3)
10-50	6.44	24 (1.6)	7 (2.7)	6† (3.3)	1 (3.6)	36 (5.0, 3.9)	2 (5.7)	24 (6.7)

* In these instances, the values represent total γ- or β-globulin, since the individual components could not be resolved accurately.

† Anomaly.

day. The hypoproteinemic animals were fed a diet containing 10 per cent casein for 160 to 261 days prior to the analyses.

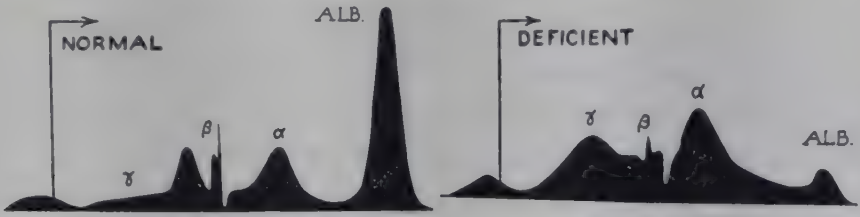


FIG. 1. Electrophoretic patterns of the sera of normal (Pig. 10-58) and protein-deficient (Pig. 10-40) swine. The deficient animal shows a marked diminution of albumin and large relative increases of the globulin components. The photographs were taken of the descending boundaries at 250 minutes. The runs were performed at 1° in veronal buffer of pH 8.4 to 8.6 and at an ionic strength of 0.1.

TABLE II

Protein Composition of Sera of Normal and Protein-Deficient Swine

The values are calculated from the percentage for each component of the total refractive increment and the total protein content of the serum with the factor 6.7 × N. The concentrations are in gm. per 100 ml. of serum.

Pig No.	Total protein	γ_2	γ_1	β_2	β_1	$\alpha_2 + \alpha_3$	α_1	Albumin
				Controls				
10-57	5.85	0.70	0.53	0.29	0.29	0.94	0.23	2.86
10-58	5.55	0.50	0.72	0.28	0.11	1.05	0.22	2.66
10-59	7.60	0.99	0.91	0.38	0.23	1.52	0.23	3.34
10-16	5.54		0.72*		0.89*	1.27	0.33	2.33
10-17	8.95	1.88	0.63	0.36	0.36	1.79	0.36	3.58
Mean..	6.70	1.52		0.64		1.58		2.95
				Protein-deficient				
10-40	3.27	0.92	0.20	0.10	0.20	1.27	0.33	0.26
10-41	4.98		1.15*	0.30	0.35	1.89	0.40	0.90
10-44	5.10	1.02	0.61		0.15*	1.99	0.46	0.87
10-45	3.74		0.86*	0.34	0.30	1.35	0.22	0.67
10-50	4.51	1.08	0.32	0.27	0.05	1.62	0.09	1.08
Mean..	4.32	1.23		0.41		1.92		0.76

* In these instances, the values represent total β- or γ-globulin; the individual components could not be accurately resolved.

The electrophoretic analyses were made on sera at 1° in a Tiselius apparatus equipped with the Longsworth schlieren scanning device. The sera were dialyzed for 48 hours against diethyl barbiturate (veronal) buffer at pH 8.4 to 8.6 and at an ionic strength of 0.1. The protein concentration in the cell was about 1.5 per cent. Only descending patterns were measured.

In Table I our studies of the serum proteins of five normal and five hypoproteinemic swine are presented. The values for the distribution and mobilities of the various components in the normal animals are in satisfactory agreement with those of previous workers (2–5). The degree of hypoproteinemia is indicated in a more marked fashion by the amount of albumin than by the serum N values. The average albumin concentration in the hypoproteinemic animals was 17 per cent as compared with 45 per cent for the controls. The relative amount of α-globulin increased strikingly. In the control animals the total globulins averaged 24 per cent and in the deficient animals 44 per cent. A moderate increase in γ-globulins from 22 to 29 per cent was noted, but there was no change in the relative amount of β-globulin. These changes are strikingly illustrated in Fig. 1 where the patterns obtained with the serum of a normal and of a hypoproteinemic animal are shown for comparison.

In Table II the absolute concentration in gm. per 100 ml. of serum for each of the serum constituents has been computed by multiplying mg. of protein nitrogen by 6.7 (9). The data show a marked diminution in albumin from 2.95 gm. per cent in the controls to an average of 0.76 gm. per cent for the hypoproteinemic swine. There was only a slight increase in the absolute amount of α-globulin and a moderate diminution in β- and γ-globulins.

Electrophoretic analyses of the plasma of these animals revealed no significant differences between the groups in the mobility or quantity of fibrinogen. The mean content for each group was about 4 per cent with a range from 3 to 6 per cent.

DISCUSSION

Our results confirm those of Zeldis *et al.* (6) and Chow (7); namely, that a prolonged dietary restriction of protein results in a marked diminution in both the relative and absolute amount of albumin, while a relative increase occurs in globulin, especially α-globulin. Previous electrophoretic analyses from this laboratory (10) on swine deficient primarily in tryptophan have revealed similar changes, although some alteration in the composition of the albumin was noted as indicated by a broad asymmetrical curve when acid-hydrolyzed casein was fed in place of crude casein.

It is of considerable interest that the degree of hypoproteinemia in these animals was much greater as determined by electrophoresis than by the chemical method (1). It is also striking that, although there was a slight reduction in the β-globulin fraction as measured electrophoretically, the metal-binding protein (β_1-globulin Fraction IV-7 (11)) as determined by the total iron-binding capacity was reduced about 65 per cent (1).

SUMMARY

Electrophoretic analyses have been performed on the sera of swine made hypoproteinemic by prolonged restriction of the dietary intake of protein and the results have been compared with the sera from control animals.

Chronic protein depletion in these animals results in a marked decrease in the absolute as well as relative amount of albumin and in a relative increase in the globulin fraction, especially in the α-globulins.

BIBLIOGRAPHY

1. Cartwright, G. E., and Wintrobe, M. M., *J. Biol. Chem.*, **176,** 571 (1948).
2. Svensson, H., *J. Biol. Chem.*, **139,** 805 (1941).
3. Moore, D. H., *J. Biol. Chem.*, **161,** 21 (1945).
4. Deutsch, H. F., and Goodloe, M. B., *J. Biol. Chem.*, **161,** 1 (1945).
5. Koenig, V. L., and Hogness, K. R., *Arch. Biochem.*, **9,** 119 (1946).
6. Zeldis, L. J., Alling, E. L., McCoord, A. B., and Kulka, J. P., *J. Exp. Med.*, **82,** 157 (1945).
7. Chow, B. F., *Ann. New York Acad. Sc.*, **47,** 297 (1946).
8. Bieler, M. M., Ecker, E. E., and Spies, T. D., *J. Lab. and Clin. Med.*, **32,** 130 (1947).
9. Armstrong, S. H., Jr., Budka, M. J. E., Morrison, K. C., and Hasson, M., *J. Am. Chem. Soc.*, **69,** 1747 (1947).
10. Cartwright, G. E., Wintrobe, M. M., Buschke, W. H., Follis, R. H., Jr., Suksta, A., and Humphreys, S., *J. Clin. Invest.*, **24,** 268 (1945).
11. Cohn, E. J., *Blood*, **3,** 471 (1948).

STUDIES ON ADENOSINETRIPHOSPHATASE OF MUSCLE*

II. A NEW MAGNESIUM-ACTIVATED ADENOSINETRIPHOSPHATASE

By W. WAYNE KIELLEY† and OTTO MEYERHOF

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)

(Received for publication, July 1, 1948)

After Engelhardt and Lyubimowa (4) announced in 1939 that the ATPase[1] activity of muscle was contained in the myosin fraction, interest centered around the question of whether the enzyme was identical with the bulk of the myosin, was only a part of it, or was only adsorbed to it (see (1)). It was taken for granted by all investigators in this field that practically all of the ATPase activity was in this fraction. According to Needham (5), less than 10 per cent of the ATPase in muscle is water-soluble, the remaining activity being bound to the insoluble residue, the myosin fraction.

An evaluation of the experiments of Lohmann (6), who was the first to study the ATPase activity of muscle, showed that the water extract at neutral pH and in the absence of Ca can dephosphorylate ATP at a rate corresponding to more than 10 per cent of the activity of the whole muscle. However, this activity quickly disappears in about an hour at room temperature, and was therefore overlooked by all later authors.

We set about the task of isolating the enzyme fraction responsible for the activity of the fresh muscle extract. In spite of the instability of the enzyme this task was relatively successful. Although the purification has so far yielded preparations which, measured in absolute activity under optimal conditions, are not more concentrated than the best fractions obtained from the myosin enzyme (1), the purified enzyme can easily be distinguished from the latter by its different pH optimum, its specific activation by Mg and inhibition by Ca, and its different stability at various temperatures.

---

* This work was aided by grants from the American Cancer Society, recommended by the Committee on Growth of the National Research Council, and the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service.

For Paper I of this series see (1); preliminary notes (2) and (3).

† Postdoctorate Research Fellow, National Institute of Health, Bethesda, Maryland.

[1] Abbreviations used, ATP = adenosine triphosphate; ADP = adenosine diphosphate; ATPase = adenosinetriphosphatase, splitting the first labile P group; pyro-P = 7 minute P minus direct P.

If both enzymes are compared under their optimal conditions of pH, activation, etc., characteristics in which they differ so greatly, the total yield measured by their activities is about equal. But under the conditions prevailing in the living muscle, neutral pH, high content of Mg, and absence of Ca in the interior of muscle, the new Mg ATPase is surely responsible for most of the dephosphorylation. In two respects it resembles the other ATPases, which so far have been obtained not only from muscle but from many other mammalian tissues: first, only the first labile P group of ATP is split off; secondly, it is a high molecular weight protein of globulin nature, forming complexes or adsorption compounds with the particulate matter, from which it cannot be easily separated.

For comparison with the results of other authors we followed the proposal of Bailey (7) and calculated the activity as Q_P; *i.e.*, microliters of H_3PO_4 split off per mg. of protein per hour at 38°. The protein was calculated from the nitrogen content with the factor 6.25. Incubations were for 5 minutes and phosphate is expressed as micrograms of P.

$$Q_P = \frac{\text{micrograms P} \times 22.4 \times 60}{\text{mg. N} \times 6.25 \times 31 \times 5}$$

Preparation of Mg ATPase—The animals (rats) were anesthetized with nembutal and killed by exsanguination. The carcasses were then chilled in ice prior to removal of the muscles of the hind leg. The muscle was minced with scissors, suspended in 6 volumes of cold extracting solution (0.5 M KCl, 0.03 M $NaHCO_3$, 0.02 M Na_2CO_3, 0.001 M KCN), and ground in a Waring blendor for about 1 minute. The suspension was allowed to stand for about 20 minutes and then centrifuged at 4000 R.P.M. for 15 minutes. The residue was reextracted with the same volume of extracting solution as before. After 15 minutes extraction this suspension was centrifuged and the extracts combined. To remove the actomyosin the combined extracts were diluted with 6 volumes of cold 0.001 M KCN and the pH adjusted to 8.0 to 8.5. After standing about 30 minutes the suspension was centrifuged and the precipitate of actomyosin discarded. The enzyme was precipitated from the supernatant by addition of $(NH_4)_2SO_4$ to 35 per cent saturation (27 gm. of $(NH_4)_2SO_4$ per 100 ml.). The pH here and in the subsequent reprecipitations was maintained at about 8.0 by addition of NH_4OH.

The $(NH_4)_2SO_4$ precipitate was centrifuged and then dissolved in 0.5 M KCl. The solution was centrifuged and the insoluble material discarded. The enzyme was then reprecipitated three times at 35 per cent saturation with $(NH_4)_2SO_4$. After each precipitation and resolution the preparation was clarified by centrifuging. After the last precipitation, the preparation was dissolved in a solution whose final composition was 0.06 M histidine, 0.4 M KCl, 0.001 M KCN. The pH should be about 7.5 at room temperature.

Starting with 25 gm. of muscle, at the dilution step at which the actomyosin was removed, the volume was somewhat greater than 2 liters. Since the handling of larger volumes was inconvenient, this was the usual weight of muscle employed. The final solution of the enzyme was then kept at 50 ml. and a 0.2 ml. aliquot of a 1:9 dilution of this solution gave 30 to 50 γ of P in the procedure for determining enzyme activity.

Though distilled water or dilute bicarbonate would extract some of the enzyme from muscle, the use of the stronger salt solution improved the yield considerably. However, since practically all of the muscle substance had been brought into suspension, it was necessary to remove the myosin or actomyosin. This was accomplished by dilution to an ionic strength of approximately 0.08. During the extraction and other manipulations the pH was maintained above 8. It has been found that up to the point of removal of the actomyosin the amount of enzyme obtained falls off sharply below pH 7.5. The amount of enzyme obtained in the $(NH_4)_2SO_4$ precipitation is also somewhat greater in alkaline solution.

When the procedure given here is followed, the quantity of enzyme obtained, when related to the weight of muscle, is sufficient to split off about 5 mg. of P per gm. of muscle in 5 minutes at 38°. By reextracting the actomyosin precipitate this may be increased by about 50 per cent, but the volumes of liquid to be dealt with do not make it worth while.

The enzyme may be further purified by high speed centrifugation. For this purpose the final solution of the enzyme was diluted with an equal volume of 0.5 M KCl and centrifuged at 10,000 × g for 20 to 30 minutes. The precipitate obtained usually contained about 15 to 20 per cent of the total activity but was generally discarded, for, though its purity was slightly greater than that of the original solution, it was considerably lower than that of the material obtained by subsequent centrifugation of the supernatant at higher speed.

The supernatant from this first centrifugation was then centrifuged at 18,000 × g for 20 minutes, and the supernatant was poured off and recentrifuged, while the precipitate was resuspended. This process was repeated until no more precipitate was obtained. Resuspension of the clear brown gel, which forms the pellets, is somewhat easier when repeated short periods of centrifugation are used rather than one period of an hour or more. The resuspended precipitates were all combined and completely dispersed by use of a hand homogenizer. As before, the solution was in 0.4 M KCl, 0.06 M histidine, and 0.001 M KCN. Finally, the preparation was centrifuged at 4500 R.P.M. for about 30 minutes and the precipitate rejected. This solution contained about 60 per cent of the total activity of the original solution before high speed centrifugation. High speed centrifugation resulted in a 3-fold increase in purity on the average. The Q_P values were usually in the range of 6000 to 10,000. Some typical results are given in Table I. Some preparations have been subjected to a second centrifugation at 18,000 × g but the Q_P values were rarely improved by as much as 50 per cent.

The concentrated preparations of the enzyme are brown in color, usually somewhat pink, and are highly opalescent. Since the sedimentation at 18,000 × g would identify the material as the small particles or microsomes obtained from various tissues by Claude (8), it is not surprising that our preparations contain about 30 per cent lipide on the dry weight basis. They have a high phosphorus content, most of which is lipide P (85 to 90 per cent). There is a small variable amount of ribonucleic acid (0 to 10 per cent). A study of the distribution of phosphorus according to the method of Schmidt and Thannhauser (9) also indicates that about 5 to 10 per cent of the P is "phosphoprotein P;" the presence of desoxyribonucleic acid is questionable. Claude has suggested that the positive Schiff test given by his preparations, which we have observed also, is due to the presence of Feulgen's acetal phospholipides (10). The nucleic acid present in our preparations can be removed with ribonuclease without influencing the enzyme activity.[2] Attempts to separate the enzyme from the lipide material have been unsuccessful.

TABLE I

Increase of Activity by High Speed Centrifugation

Initial (solution of $(NH_4)_2SO_4$ ppt.), Q_P	Centrifugation at 18,000 × g, Q_P	Increase × initial
2860	8,100	2.8
2790	7,350	2.6
3170	10,960	3.5
2410	7,360	3.1
2940	8,600	2.9
2270	6,700	3.0

Though the preparations are quite unstable, the loss of activity is retarded by the presence of cyanide and some preparations have even been observed to increase in activity for a few days. In the case of two preparations this extended over a period of 8 days and the activity doubled in this time. However, these magnitudes were uncommon and the preparations usually did not retain their activity more than 7 or 8 days.

---

[2] We thank Dr. Kunitz for supplying a sample of crystalline ribonuclease.

In the clarification following each $(NH_4)_2SO_4$ precipitation, a black precipitate was obtained. On the basis of a pronounced test for Fe, this was presumed to be hemin. This fact, coupled with the appearance of the preparations, suggests that the cyanide effect is through formation of complexes with Fe and possibly other heavy metals.

The pH of maximum stability (about 7.5) is somewhat higher than the pH optimum of the enzyme. This is probably due to the effect of pH on CN^- concentration and loss of HCN from the solution.

Preparation of Myosin—The preparation of "crystalline myosin" given by Szent-Györgyi (11) was employed. It was found that myosin binds phenolphthalein rather strongly and, therefore, a pH meter was used rather than the indicator in that part of the procedure in which residual actin is removed.

Preparation of Actin—Bailey and Perry (12) found that the turbidity of actin prepared by the modified method of Straub (11) could be reduced by chloroform extraction of the acetone-dried muscle residue. We have made similar observations, using alcohol-ether extraction. Otherwise the preparation was identical with that of Straub.

Preparation of ATP—Our preparations, made from rabbit muscle, followed the procedure of Kerr (13) with modifications (1). For preparing a neutral stock solution, Ba and heavy metals were removed by Amberlite resin. A 30 × 0.8 cm. tube was filled with 15 gm. of Amberlite (washed with Na_2CO_3) and 250 mg. of Ba salt dissolved in 0.1 N HCl were washed through during about 30 minutes. The total volume was kept at 25 ml. and the final pH was 7.0 to 7.4. The solution could be kept in the ice box for weeks.

The amount of ADP in the ATP solution is usually determined by the ratio of 7 minute P to total P. A more accurate method consists of incubating an aliquot of the ATP solution with an excess of glucose and purified hexokinase from yeast. The transphosphorylation to glucose stops when the first labile P group has been transferred.

However, the hexokinase preparation must be free of phosphohexoisomerase, or a correction must be applied for the fructose-6-phosphate formed. This correction amounts to 5 per cent of the 7 minute P, if the equilibrium of isomerization is attained. A once crystallized preparation of hexokinase, kindly supplied by Dr. Kunitz, still contained some isomerase, which necessitated a correction of 1.5 per cent. By this method, our own preparations, freshly made, contained 90 to 96 per cent ATP, while the remaining 4 to 10 per cent of the labile P was ADP. A commercial preparation of the Na salt from Rohm and Haas, Philadelphia, contained as much as 28 per cent ADP; tested with two different preparations of the Mg ATPase, 35.5 per cent of the total 7 minute P was split off, an excellent agreement (see

Fig. 1). It should be noted that with the usual procedure for developing the color of molybdenum blue, in the modified Fiske-Subbarow procedure for phosphorus, 2 per cent of the pyro-P is split. This inaccuracy can be avoided if the color is developed in the presence of ethyl alcohol (14) and then immediately read in an Evelyn colorimeter.

Measurement of Activity—Activity was determined in a system consisting of 1.0 ml. of buffer (0.1 M histidine or 0.05 M borate), 0.1 ml. of 0.15 M $MgCl_2$ or $CaCl_2$, 0.5 ml. of ATP (600 to 700 γ of 7 minute P per ml.), a suitable aliquot of the enzyme solution (usually 0.2 ml.), and H_2O to a total volume of 3.0 ml. Incubations were made at 38° for 5 minutes and the reaction stopped with 3.0 ml. of 5 per cent trichloroacetic acid. The solutions were then analyzed for inorganic phosphorus with the methods used in this laboratory.

Protein Determination—Nitrogen was determined by micro-Kjeldahl analysis according to the method of Ma and Zuazaga (15). Since the preparations contained ammonia as well as histidine, the protein was first precipitated with 10 per cent trichloroacetic acid, then twice resuspended in dilute trichloroacetic acid, and finally dissolved in N NaOH and transferred to the digestion flasks.

pH determinations were made with a Cambridge model L pH meter.

Factors Influencing Activity of Mg ATPase

Substrate Specificity—The Mg ATPase has no effect on β-glycerophosphate, hexose diphosphate, adenylic acid, and ADP. In the decomposition of ATP the reaction stops when one phosphate group has been removed. There appears to be no suppression of the activity by the accumulating ADP and the decomposition follows a curve for a first order reaction. Fig. 1 shows a plot of the data from one experiment. The curve was drawn on the basis of a total substrate concentration of 113 γ of P derived from the hexokinase analysis of the ATP solution. The rate constant was obtained from the data of two different enzyme preparations.

Influence of pH—The relationship between pH and activity of the Mg ATPase is shown in Fig. 2. The pH optimum is about 6.8 at 38°. On the acid side of the isoelectric point of histidine the pH of the buffer is reduced about 0.20 to 0.25 pH unit for a 10° rise in temperature. The effect of temperature on borate buffers is negligible.

The selection of a suitable buffer in the region of the pH optimum of the Mg ATPase presented some difficulties. The phosphate buffers ordinarily used around neutrality obviously could not be used here. The only other buffer in general use, acetate-veronal, has practically no buffer capacity in the region of pH 5.5 to 7.0. Though not generally used as a buffer, histidine is quite good throughout the range of pH 5 to 11. The buffer solution was made up in 0.1 M concentration with the ionic strength adjusted to 0.075 with KCl. There is no indication that the choice of buffer influenced the results as long as the pH was maintained.

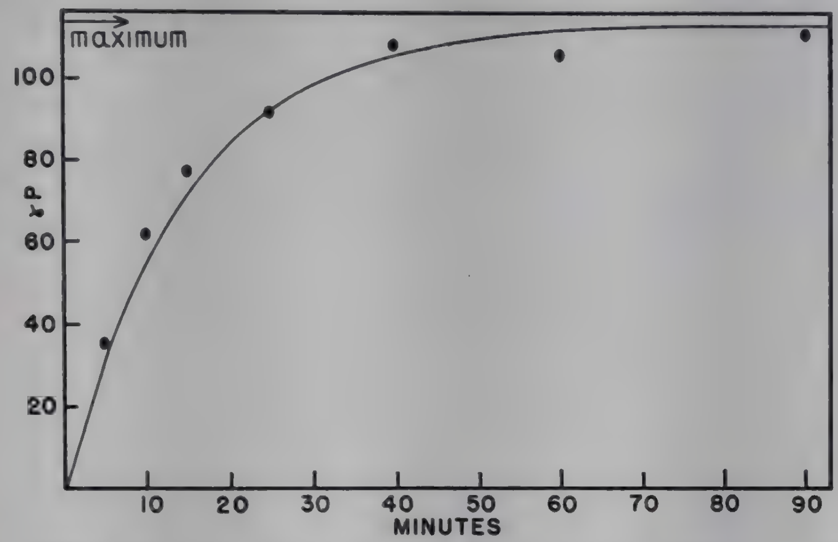


FIG. 1. ATP hydrolysis catalyzed with Mg-activated ATPase. 0.00736 mg. of protein nitrogen in 3 ml. Mg concentration = 0.005 M. First P group of ATP given by hexokinase analysis equivalent to 113 γ of P. For the curve, $k = 0.066$ min.$^{-1}$

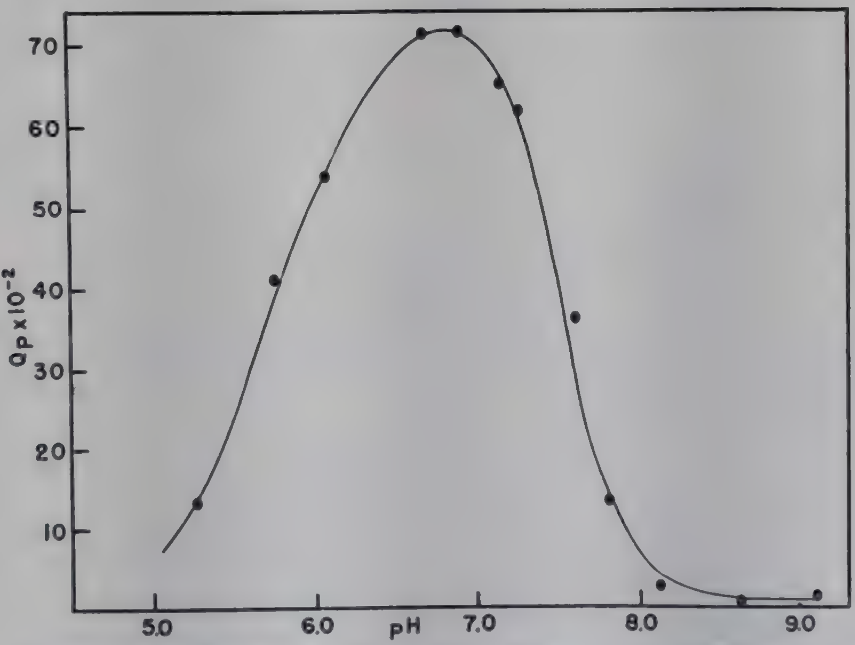


FIG. 2. Influence of pH on the activity of the Mg-activated ATPase. Incubation run in histidine buffer (0.03 M). Mg concentration = 0.005 M. Time, 5 minutes; temperature, 38°.

With myosin, on the other hand, the only consistent results have been obtained in borate buffers. The mixtures of myosin and actin, however, seem somewhat less affected by the choice of buffer, and for the Mg activation of these mixtures the pH optimum in histidine is somewhat lower than in borate.

Activation and Inhibition—The new enzyme is activated by Mg and to some extent by Mn; Ca, Ba, and Co are without effect. Ca inhibits when both Ca and Mg are present in equal concentration. The relationship between activity and concentration of Mg, Mn, and Ca is represented in Fig. 3. There is always some activity in the absence of added Mg. Estimates of the dissociation constant for an Mg-enzyme complex have varied from 3.0×10^{-4} to 1.0×10^{-3}. The presence of other ions as well as the ionic strength influences the activity to a minor degree. In the system for measuring activity, the activity is optimal at an ionic strength of about 0.1. When the ionic strength is adjusted with K_2SO_4 rather than KCl, the activity is about 10 per cent greater with $SO_4^=$ than with Cl^-.

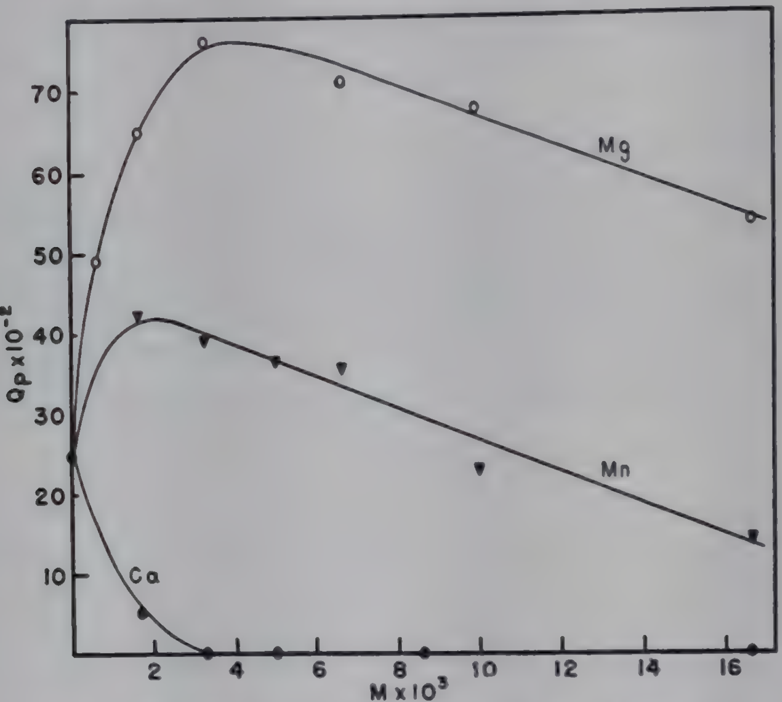


FIG. 3. Influence of Mg, Mn, and Ca on activity of Mg ATPase. Incubations in histidine buffer, pH 6.9, at 38° for 5 minutes. Total substrate equivalent to 113 γ of P (first group of ATP).

In addition to the inhibition by Ca, the enzyme is inhibited by fluoride and, judging by experiments with *p*-chloromercuribenzoate, sulfhydryl reagents will also inhibit. CN^- and N_3^- when added to the incubation mixture have a negligible effect on the activity.

Relationship between Activity, Enzyme Concentration, and Substrate Concentration—The activity is linear with enzyme concentration as long as the substrate concentration is non-limiting. The relationship between activity and substrate concentration appears to behave according to the concept of formation of an enzyme-substrate complex, though determinations of the Michaelis-Menten constant have not been highly reproducible, varying from 2.0 to 4.0 $\times 10^{-4}$.

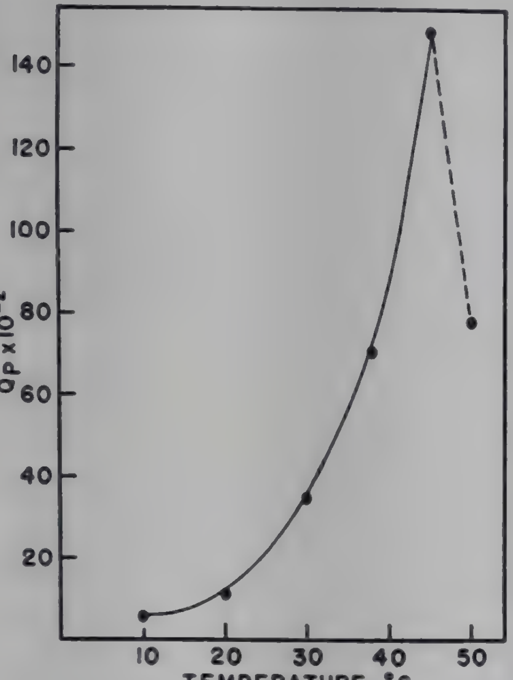


FIG. 4

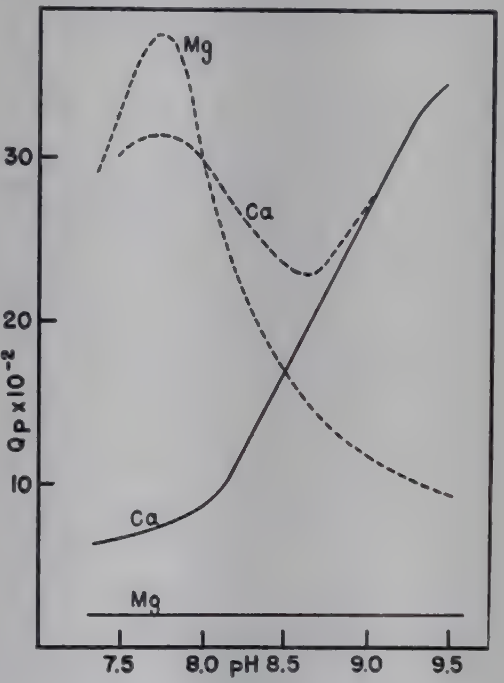


FIG. 5

FIG. 4. Influence of temperature on the decomposition of ATP catalyzed by the Mg ATPase. Histidine buffer, pH 6.9 at 38° or pH 7.5 at 10°. Mg concentration = 0.005 M; total substrate equivalent to 113 γ of P (first group of ATP).

FIG. 5. Schematic representation of enzymatic behavior of myosin and myosin + actin. Solid line, myosin; dotted line, myosin + actin. The behavior is represented for borate buffers with the ordinate in $Q_P \times 10^{-2}$.

Influence of Temperature—The relationship between activity and temperature is reproduced in Fig. 4. The temperature coefficient is in the neighborhood of 2.6. At pH 7.15 the enzyme can withstand incubation at 38° for 1 hour without loss of activity. Myosin ATPase, on the other hand, loses its activity rapidly under these circumstances.

DISCUSSION

During the course of the investigation involving the Mg-activated ATPase the question of a possible relationship with the myosin system naturally arose. Szent-Györgyi (11) noted that myosin is Mg-activated in the presence of actin. We have given particular attention to this situation in order to determine whether or not it bears any relationship to our enzyme. In addition, we have found changes in the behavior of myosin to Ca activation in the presence of actin. The shape of the activity-pH curve changes, resulting in two nearly equal maxima. The maximum around pH 9.5 does not change greatly but a new peak develops around pH 7.7 to 7.8. Though variable, in some cases this has amounted to a 5-fold increase over myosin alone at this pH. Because of the high viscosity of myosin-actin mixtures there are obvious difficulties attendant on the investigation of the enzymatic activity of these preparations. However, from the results of a large number of experiments on this system a schematic representation of the behavior has been constructed and is reproduced in Fig. 5. When histidine buffers are used rather than borate, the myosin-Ca curve is considerably depressed; the actomyosin-Ca relationship shows no alteration around pH 7.7 to 7.8 but the upper portion of the curve is depressed. The actomyosin-Mg curve is displaced, the maximum occurring around pH 7.4.

It should also be pointed out that though the activity-pH relationship of actomyosin has been specifically studied in the presence of either Ca or Mg, several experiments run at pH 7.4 indicate that the presence of these ions has little effect on the activity. This may be of considerable importance in the chemical mechanism of contraction, even though the physiological status of actin may not be clear. Though all of our observations on the myosin-actin system agree in the qualitative sense, the quantitative aspects have been quite variable and appear to depend on the individual myosin and actin preparations.

We have not been able to observe any variations in the behavior of the Mg ATPase when either myosin or actin is added to it. Nor have we been able to separate an Mg-activated component from myosin-actin mixtures. When the procedure used in isolating the Mg ATPase was applied to myosin-actin mixtures, the activity in the presence of Ca was completely recovered in the actomyosin, but a considerable portion of the activity in the presence of Mg was unaccounted for.

SUMMARY

An unstable Mg-activated ATPase has been isolated from muscle by extraction with dilute alkaline solution and repeated fractionation with 0.35 per cent saturated ammonium sulfate. The enzyme is bound to particulate matter which is centrifuged at high speed. It is free of myosin and actomyosin.

The enzyme splits off only one labile P group of ATP. It has a pH optimum at 6.8 and is strongly inhibited by Ca. The activity is around

Q_P 8000, and the total yield under optimal conditions is about equal to that of the myosin ATPase. So far no indications have been found that it is another form of the latter enzyme.

BIBLIOGRAPHY

1. Polis, B. D., and Meyerhof, O., *J. Biol. Chem.*, **169,** 389 (1947).
2. Kielley, W. W., *Federation Proc.*, **7,** 164 (1948).
3. Kielley, W. W., and Meyerhof, O., *J. Biol. Chem.*, **174,** 387 (1948).
4. Engelhardt, W. A., and Lyubimowa, M. N., *Nature*, **144,** 669 (1939).
5. Needham, D. M., *Biochem. J.*, **36,** 113 (1942).
6. Lohmann, K., *Biochem. Z.*, **271,** 264 (1934).
7. Bailey, K., *Biochem. J.*, **36,** 121 (1942).
8. Claude, A., *Science*, **97,** 451 (1943).
9. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **161,** 83 (1945).
10. Feulgen, R., and Bersin, T., *Z. physiol. Chem.*, **260,** 217 (1939).
11. Szent-Györgyi, A., Chemistry of muscular contraction, New York (1947).
12. Bailey, K., and Perry, S. V., *Biochim. et biophys. acta*, **1,** 506 (1947).
13. Kerr, S. E., *J. Biol. Chem.*, **139,** 121 (1941).
14. Meyerhof, O., and Oesper, P., *J. Biol. Chem.*, **170,** 1 (1947).
15. Ma, T. S., and Zuazaga, G., *Ind. and Eng. Chem., Anal. Ed.*, **14,** 280 (1942).

PREPARATION AND CHARACTERIZATION OF DEXTRAN FROM LEUCONOSTOC MESENTEROIDES

By ALLENE JEANES, C. A. WILHAM, AND J. C. MIERS*

(*From the Starch and Dextrose Division, Northern Regional Research Laboratory, Peoria, Illinois*†)

(Received for publication, June 21, 1948)

In conjunction with investigations on the α-1,6-glucosidic linkage in starch, it was desirable to conduct comparative studies on other substances containing this linkage. The dextran from *Leuconostoc mesenteroides*, a polysaccharide having predominantly α-1,6-glucosidic linkages, was selected to be used directly in these comparative studies and to provide a source of simpler substances containing this linkage of rare occurrence. Experiments were designed to provide a dependable source of dextran of high purity and of reproducible high viscosity. Improvements have been made over methods previously described for the preparation of this polysaccharide (1–4), and procedures have been established for the preparation of dextrans of low as well as of high viscosities.

Factors Influencing Dextran Production—Numerous factors, only a few of which have been studied (1, 4), appear to influence the properties and the amount of the dextran and of the by-products produced from sucrose by cultures of *Leuconostoc mesenteroides*. A factor of outstanding importance is the strain of the organism (5), which appears to determine whether the dextran is water-soluble or water-insoluble (3, 6). The structural basis for this difference in solubility is not known. Previously reported dextrans, most of which originated from different strains of *Leuconostoc mesenteroides*, have varied in other physical properties. Dextran has been obtained in yields of 18 (3) and 25 per cent (4), with specific rotations of +178° to +184° (2, 6, 7), of +195° (8), and of +198° (9). It has been reported to have high (2, 4, 6) and low (1, 9) contents of nitrogen, phosphorus, and ash. Some viscosity data for dextran are not on a comparable basis (7, 8) and other data present unexplained variations (5).

The optimum pH range for the enzymatic synthesis of dextran has been shown by Hehre to be 4.0 to 6.0 (7, 10). When preparing dextran from cultures of *Leuconostoc mesenteroides*, neutralization of the acidic by-products has been reported to increase the yield of dextran (1, 4, 11).

The incubation time used previously for the production of dextran has varied from 18 hours to 20 days (1–4). Methods reported for determining when dextran formation was complete have involved isolating and weighing the dextran from an aliquot of the medium (1) and measuring the viscosity of the culture medium (12).

---

* Present address, Western Regional Research Laboratory, Albany, California.

† One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

In the present investigation, the data obtained are for *Leuconostoc mesenteroides* NRRL B-512 and its water-soluble dextran. Observations are reported here on the effect of pH and the composition of the culture medium, aeration, and incubation time on the yield and properties of this dextran. The manner of inoculation has been held constant.

Production of High Viscosity Dextran—The viscosity of dextran from *Leuconostoc mesenteroides* NRRL B-512 was found to be influenced greatly by cultural conditions. However, when these conditions were controlled, dextrans of high or of low viscosity were obtained as desired.

The procedure adopted for the preparation of dextran utilized an unaerated medium containing sucrose in 10 per cent concentration and buffered only with the mineral nutrients present. This was inoculated heavily with a rapidly growing culture of *Leuconostoc mesenteroides* NRRL B-512 and incubated at 25°. The course of dextran production was followed by measurement of the viscosity of the culture medium. The formation of dextran was paralleled by an increase in viscosity until, after about 24 hours incubation, the viscosity reached a maximum and dextran formation appeared to be completed. As is shown in Table I, these changes were accompanied by a decrease in pH from the initial value of about 7.0 to about 4.6 at the time of maximum viscosity.

Dextrans such as Dextrans A and D of Table II, which were isolated from the culture media at maximum viscosity, are called high viscosity dextrans. These dextrans after purification had characteristically high viscosities, highly positive optical rotations, and high purity.

The reproduction of results from preparations of high viscosity dextrans is demonstrated by Dextrans A and D (Table II) and by another typical high viscosity dextran which had a relative viscosity of 2.230 and was obtained in 26.8 per cent yield. Dextran B is not a typical high viscosity dextran (see Table I, foot-note).

In all cases, the products called dextrans were quantitatively precipitated as gummy masses by the addition of an equal volume of absolute ethanol to the culture medium. The technique used for isolating purified dextrans from aqueous solutions and for drying the product gave finely divided fluffy dextrans, which dispersed readily in water and underwent chemical reaction with ease when in the dry state.[1]

Production of "Autolyzed" Dextran—The viscosity of the culture medium containing dextran decreased when incubation was extended beyond the time of maximum viscosity. This decrease in viscosity, as is shown in Table I, was rapid at first and gradually became slower until a very low value was reached. During this incubation period, the pH of the culture medium decreased slowly from 4.6 to a steady value of 3.7.

---

[1] Jeanes, A., and Wilham, C. A., in preparation.

Dextrans such as Dextrans C and E of Tables I and II, which were isolated from culture media of greatly reduced viscosity, are called "autolyzed" dextrans. In physical properties the "autolyzed" dextrans differ from the corresponding high viscosity dextran controls (Dextrans B and D) mainly in their lower viscosities. The viscosities of the "autolyzed" dextrans were inversely proportional to the duration of incubation; their yields were 2.1 to 2.5 per cent lower than those of their high viscosity controls.

TABLE I

Effect of Extended Incubation at 25° on pH and Viscosity of Unbuffered Culture Media

Culture Medium BC				Culture Medium DE			
Incubation time	pH	Absolute viscosity	Fraction isolated	Incubation time	pH	Absolute viscosity	Fraction isolated
hrs.		*centipoises*		*hrs.*		*centipoises*	
0	7.5	2		0	7.1	2	
23	4.9	60		23	4.6	203	Dextran D*
29	4.5	82		27.5	4.4	164	
32	4.45	75		32	4.3	131	
33	4.4	73	Dextran B*				
				47	4.0	74	
				72	3.9	43	
99	3.85	20		96	3.75	20	
				145	3.73	15	
191	3.7	11					
				273	3.7	10	Dextran E and other fractions
335	3.7	7					
384	3.7	6					
503	3.7	5	Dextran C and other fractions				

* These dextrans were isolated from one-half of their respective culture media, and the incubation of the other half was continued. When the isolation of Dextran B was actually begun, the viscosity of this part of the culture medium had decreased to 59 centipoises.

Production of Dextran in Aerated and in Buffered Culture Media—Aeration of culture media was found to be unfavorable to dextran formation. Data on the pH and viscosity changes in an aerated, unbuffered culture medium are given in Table III, and the data on the product isolated therefrom, Dextran F, are given in Table II. As compared with the results from unaerated culture media, aeration decreased the rate of formation, the yield, and the viscosity of the dextran, and did not prevent the culture medium from passing through a maximum viscosity. These results are not in conflict with the view of Hehre (10) that the action of the dextran-synthesizing enzyme does not appear to be "coupled with or dependent upon oxidative processes."

TABLE II

Data on Dextrans from Various Culture Media

Dextran	Absolute viscosity of culture medium from which dextran was isolated	Properties of purified dextrans					
		Yield	N	P	Relative viscosity at 25°, 0.5 per cent in water*	$[\alpha]_D^{25}$ (in 1 N NaOH, $C = 1$)	Alkali No.†
	centipoises	*per cent*	*per cent*	*per cent*		*degrees*	
A	446	25.3‡	0.017	0.005	2.253	+203	0.0
B	73	24.7	0.033	0.008	2.003	201	0.0
C	5	22.2	0.022	0.011	1.414	199	0.6
D	203	23.7	0.032	0.007	2.133		0.1
E	10	21.6	0.010	0.004	1.565	200	0.3
F	14	<14.7			1.855	200	
G	847	24.0‡	0.000	<0.002	1.719	202	0.0

* The relative viscosities in 0.1 M calcium acetate of Dextrans A, C, and G were 2.235, 1.405, and 1.683, respectively. In 1.0 M calcium acetate, the value for Dextran A was 2.298.

† To serve as a basis for comparison, the following alkali numbers are quoted from (13): defatted corn-starch 11.0; corn amylose 20.2; corn amylopectin 5.9.

‡ The ash content of these representative dextrans was 0.02 per cent.

TABLE III

Effect of Aeration on pH and Viscosity of Unbuffered Culture Medium

Operation	Incubation time	pH of culture medium	Absolute viscosity of culture medium
	hrs.		*centipoises*
Aeration started*	0	7.5	2
" stopped	23.5	5.2	5
	32	4.7	12
	47.5	4.4	16
Dextran F isolated	55	4.3	14

* About 20 liters of air per hour were bubbled through the 6 liters of culture medium containing a small amount of lard oil to prevent foaming.

No advantage to dextran formation has been found by buffering the culture medium with calcium carbonate. Culture media buffered at pH

7.0 to 6.4 with calcium carbonate reached their maximum viscosity in 48 to 56 hours, and immediately the viscosity began to decrease rapidly. A dextran isolated from such a medium at its maximum viscosity is Dextran G of Table II. Comparison with the corresponding values for unbuffered preparations shows that buffering with calcium carbonate resulted in a marked increase in the viscosity of the culture medium, no increase in the yield, and a decrease in the viscosity but no decrease in the purity of the purified dextran.

Aerated media buffered with calcium carbonate did not pass through a maximum viscosity; the viscosity of such a medium increased slowly over a period of 30 days. Under these conditions the rate of dextran formation was decreased as compared with an unaerated, buffered medium.

Other Polysaccharide Fractions—In addition to dextran, culture media of *Leuconostoc mesenteroides* NRRL B-512 contained other polysaccharides in small amounts which were precipitated by adding ethanol to the media to make ethanol concentrations of 65 and 75 per cent. The amounts and properties of the fractions obtained varied with the conditions of production, but all fractions appeared to contain combined fructose. Some of the fractions were levans, and some became insoluble in water after one precipitation.

It has not been reported previously that *Leuconostoc mesenteroides* produces both dextran and levan. A few strains of *Streptococcus bovis* and of *Streptococcus salivarius* have been observed to produce both levans and dextrans (14).

EXPERIMENTAL

The medium used for growth of *Leuconostoc mesenteroides* NRRL B-512 and for the preparation of dextran was the same as that used by Hassid and Barker (2), except that 0.1 per cent of sodium chloride was added, as recommended by Tarr and Hibbert (1). The dipotassium hydrogen phosphate, in 5 per cent solution, was sterilized separately and added aseptically to the cool, sterile solution of the other components. Sterilization was effected by autoclaving at 15 pounds per sq. in. for 30 minutes.

Preparation of Inoculum—A culture of the organism was prepared by inoculating one standard loopful of rapidly growing stock culture into 125 cc. of sterile medium contained in a 300 cc. Erlenmeyer flask. This was shaken mechanically for 24 hours at 25°, and then transferred to 500 cc. of medium in a 3 liter Fernbach flask. After standing for 24 hours at 25°, this culture, totaling 625 cc., was transferred to 3 liters of the medium for the preparation of dextran.

The preferred incubation time of 24 hours for the 625 cc. inoculum was adopted on the basis of experimental observations. When 24 hour inocula were used, culture media for dextran production reached high maximum viscosities such as that of the typical Dextran A of Table II. When 36 or 48 hour inocula were used, as for Dextrans D and B, respectively, progressively lower maximum viscosities of the culture media were obtained (Table II). It appears that the incubation time for the 625 cc. inocula influenced the viscosity of these unbuffered culture media.

Preparation of High Viscosity Dextran—12 liters of medium were sterilized in a 20 liter Pyrex bottle which was equipped with a siphon for the aseptic withdrawal of test samples. Incubation was at 25°. The pH values for this preparation culture were 7.1 after inoculation, 4.95 at 24 hours incubation time, and 4.75 at 26 hours. At incubation times of 24 and 26 hours the absolute viscosity of the medium was 438 and 446 centipoises, respectively. The viscous material appeared to be homogeneously dispersed in the cloudy culture medium and did not settle out.

Supercentrifugation of the culture medium was started at the end of 26 hours incubation. The residue consisted largely of bacterial cells. Absolute ethanol to make 35 per cent by volume was stirred into the centrifugate, and the solution was again passed through the supercentrifuge to remove the remaining small amount of bacterial cells. The centrifugate was stirred mechanically while the ethanol concentration was made up to 50 per cent by volume. The dextran separated as a gummy mass from which the supernatant was decanted. The dextran was kneaded to remove mother liquors, and was washed three times by kneading with 50 per cent ethanol. It was then dissolved in 11 liters of water and precipitated by addition of an equal volume of ethanol. The silvery looking mass was again isolated, kneaded, and washed as before. This cycle of reprecipitation and washing was repeated twice more.

The dextran, redissolved in 2.5 liters of water, was precipitated by slowly adding 100 cc. portions of the solution to 500 cc. of absolute ethanol, which was agitated in a Waring blendor. The precipitates were combined, collected on a filter, washed twice by resuspension in 4 liters of absolute ethanol, and filtered. The product was dried *in vacuo* over anhydrous calcium chloride at room temperature. The weight of the product (dry basis) was 361 gm., 25.3 per cent of the initial weight of sucrose, or 50.6 per cent of the glucose available from the sucrose. Dextran A was shown not to contain carbohydrates small enough to dialyze through Visking cellulose membranes. Other data on this product (Dextran A) are given in Table II.

Preparation of "Autolyzed" Dextran—The changes in pH and viscosity during the preparation of two "autolyzed" dextrans are given in Table I and data on the purified products are given in Table II. The time of incubation of the 625 cc. inocula was 48 hours for Culture Medium BC and

36 hours for Culture Medium DE. 6 liter quantities of culture media were used. At 33 hours incubation, when Culture Medium BC was slightly past its maximum viscosity, one-half of it was removed aseptically and the high viscosity control, Dextran B, was isolated. The remaining half of the culture medium was allowed to stand at 25° for a total of 503 hours. The product insoluble in 50 per cent ethanol, "autolyzed" Dextran C, was then isolated in the usual way. In Culture Medium DE, the high viscosity control, Dextran D, was isolated from half of the culture medium at the time of maximum viscosity, and the "autolyzed" Dextran E was isolated from the remaining half of the medium after a total incubation of 273 hours.

The viscosities of Culture Media BC and DE, although of markedly different maximum values, decreased to 20 centipoises in 90 to 100 hours. After this time, the rate of change in viscosity was about the same in both media. At about 200 hours incubation, the pH of the culture media had reached a steady value of 3.7, and from then on changes in viscosity were very slow. This seems to be a practical time to isolate "autolyzed" dextrans.

Preparation of Dextran in Presence of Calcium Carbonate—Except as otherwise stated, all conditions and manipulations were the same as have been described for unbuffered culture media.

The 625 cc. inoculum contained 2 per cent of calcium carbonate and was incubated for 48 hours at 25° with occasional shaking. When this was transferred to the medium for preparation of dextran, 2 per cent sterile calcium carbonate was also added and kept suspended by occasional swirling during incubation of the culture medium. After inoculation, this culture medium had a pH of 7.0 to 7.1, and a viscosity of 2 centipoises.

When the culture medium was not aerated, the pH values were 6.5 at 22 hours and 6.4 at 47 hours; the corresponding viscosities were 4 and 879 centipoises, respectively. At 50 hours, isolation of the dextran was started. The culture medium was diluted with about one-third its volume of water, and supercentrifuged. The pH of the centrifugate was adjusted to 4.4 with acetic acid, and absolute ethanol was added to give an ethanol concentration of 35 per cent by volume. The mixture was then passed twice through the supercentrifuge and the dextran, isolated in the usual way, gave a 24 per cent yield. Other data for this Dextran G are given in Table II. The low nitrogen and phosphorus contents indicate that the procedure for purification of this dextran was more efficient in removing bacterial cells than that described for high viscosity dextran.

When the culture medium was aerated, the viscosities at 42, 50, 66, and 72 hours incubation time were 29, 122, 580, and 768 centipoises, respectively. During this time the pH was near 5.8. After 90 hours, when the viscosity was 786 centipoises and the pH was 6.0, the dextran was isolated. The yield was 22 per cent, and the relative viscosity of the dextran was 1.666.

Effect of Sterilization on Decrease in Viscosity—An experiment was conducted to determine whether a decrease in viscosity would occur in a dextran preparation medium in which bacterial and enzyme action had been stopped by autoclaving. The usual unbuffered medium was inoculated with a 625 cc. inoculum which had been incubated 44 hours. After 24 hours incubation, the pH was 4.65 and the viscosity was 171 centipoises. The medium was autoclaved at 15 pounds per sq. in. for 30 minutes, and cooled quickly. The pH was still 4.65 and the viscosity was 138 centipoises. The pH was adjusted to 4.2 with sterile butyric acid solution, and the mixture was kept at 25° for a time which is expressed as a continuation of the incubation period. The pH remained constant, and the viscosity at 50, 121, and 174 hours was 135, 129, and 125 centipoises, respectively. Although a slow change in viscosity occurred under these conditions, the viscosity of this solution at 174 hours incubation was roughly 10 times the viscosities observed at comparable times for media in which normal autolysis had occurred (see Table I).

Effect of Variation of Medium—When the usual unbuffered medium was supplemented with 0.5 mg. of manganous sulfate monohydrate per liter (15), the incubation times for the two inocula and for the preparation culture medium were 16, 9, and 6 hours, respectively. The purified dextran, obtained in 24 per cent yield, had a specific rotation of +200.1° and a relative viscosity of 2.255. By using an unaerated, calcium carbonate-buffered medium to which 1 mg. of manganous sulfate monohydrate per liter had been added, the incubation time was 13 hours for both of the inocula as well as for the preparation culture medium. The purified dextran, obtained in 27 per cent yield, had a specific rotation of +200.8° and a relative viscosity of 1.673. Extension of the incubation time resulted in inactivation of the bacteria in the unbuffered inocula and in a decrease in viscosity of both the buffered and the unbuffered culture media.

Substitution of the mineral constituents of Dunn *et al.* (16) (with only 0.1 the concentration of ferrous and manganous sulfates recommended) for the mineral constituents of our medium resulted in no significant increase in dextran production. Inclusion in the medium of corn steep liquor, autolyzed yeast, or Bacto-tryptone appeared to be of no advantage.

Other Polysaccharide Fractions—From the 50 per cent ethanolic dextran mother liquors, after supercentrifugation to remove a small amount of dextran, fractions insoluble in 65 and 75 per cent ethanol were successively precipitated. The fractions were purified and isolated as white powders in a manner analogous to that described for dextran.

The fractions from unbuffered "autolyzed" media (see Table I) were homogeneously water-soluble, and their yields totaled 5 per cent of the initial weight of sucrose in the medium; dialysis reduced their nitrogen and phosphorus contents from about 0.04 and 0.20 per cent, respectively, to 0.02 per cent, but did not effect any significant change in other properties. These fractions had $[\alpha]_D^{25} = +55°$ to $+133°$ (in 1 N sodium hydroxide, $C = 1$), relative viscosities of 1.060 to 1.134 (0.5 per cent concentration in water, 25°), alkali numbers of 4 to 13, and contained combined fructose. Exposure in air having 100 per cent relative humidity at 25° converted them from a state which produced amorphous x-ray patterns to states from which x-ray diffraction line patterns characteristic of dextran were obtained (17).

The fractions from unbuffered, high viscosity culture media totaled about 4 per cent in yield. One fraction, isolated in 2.6 per cent yield, had $[\alpha]_D^{25} = -23°$, an alkali number of 1, and produced only amorphous x-ray patterns (17). Another fraction, which became water-insoluble during isolation, gave a dextran x-ray line pattern without further treatment.

Fractions totaling 3.4 per cent in yield were obtained from a calcium carbonate-buffered culture medium. A fraction, obtained in 2.8 per cent yield, gave $[\alpha]_D^{25} = -54°$ and an alkali number of 0.

Tests for ketose, which is assumed to be fructose, in these fractions were made by allowing some of the dry carbohydrate to stand in 85 per cent phosphoric acid at room temperature (18). Under these conditions, fructose, sucrose, inulin, and calcium 5-ketogluconate developed dark brown to black colors within 24 hours, but neither glucose nor dextran produced any color. Some fractions produced dark brown to black colors, and others produced light yellow to tan colors when tested in this way. The intensity of color appears to indicate the relative amount of combined fructose present. Further evidence of the presence of much fructose in fractions which produced dark colors was provided by their negative or low positive optical rotations.

Preparation of Water-Insoluble Dextran—For comparison with the water-soluble dextran from *Leuconostoc mesenteroides* NRRL B-512, the water-insoluble dextran from *Leuconostoc mesenteroides* NRRL B-523 was prepared. Our usual unbuffered sucrose medium was used. Because of the slowness of growth of the organism, 48 hours incubation was required. The culture medium became viscous with insoluble gelatinous particles. Microscopic examination revealed a heavy growth of non-capsulated bacteria, and discrete particles of gelatinous material.

The culture medium was diluted with an equal volume of water and centrifuged at 3300 R.P.M. The residue, which was insoluble in boiling water, was dissolved in 0.75 N potassium hydroxide. This solution was neutralized with acetic acid and supercentrifuged twice to remove bacteria.

Addition of an equal volume of ethanol precipitated the polysaccharide and rendered it insoluble in water. The precipitate was washed with 50 per cent ethanol and the polysaccharide, called Fraction J, was isolated in the usual manner as a coarsely fluffy product.

The supernatant from the diluted culture medium was supercentrifuged and an equal volume of ethanol was added. The precipitate, which swelled greatly in water but did not dissolve, was washed with 50 per cent ethanol and the polysaccharide, called Fraction K, was isolated from absolute ethanol.

The yields of Fractions J and K were 9 and 7 per cent, respectively, of the initial weight of sucrose. Fraction J gave $[\alpha]_D^{25} = +208°$ (in 1 N sodium hydroxide, $C = 1$). Both fractions gave negative tests for fructose in 85 per cent phosphoric acid. The percentage of nitrogen in Fraction J was 0.008, and in Fraction K 0.020.

Analytical Methods—Because anhydrous dextran is very hygroscopic, samples were equilibrated with moisture in a constant humidity room (50 per cent relative humidity at 25°) where all weighings were made. Under these conditions the moisture content of the dextrans was 12 to 13 per cent. The moisture content was determined on separate samples, and all results were calculated on a dry basis.

Dextran, which was always dried *in vacuo* over anhydrous calcium chloride at 25° before being equilibrated in 50 per cent relative humidity, was shown by ethoxyl determination to contain no ethanol.

Viscosity measurements were made with standardized Ostwald-Cannon-Fenske viscosimeter tubes, at 25° ± 0.03°. For measurements on purified dextrans, 0.5 per cent aqueous solutions were used after filtration through fritted glass funnels to remove traces of lint. For measurements on culture media, care was taken to obtain representative samples, and samples from calcium carbonate-buffered media were filtered through fritted glass funnels before use.

Optical rotations were read with the light from a sodium vapor lamp. Solutions in 1 N sodium hydroxide were used to avoid the opalescence which high viscosity dextrans gave in water solutions. However, specific rotations in water were only a few degrees lower than those in sodium hydroxide.

Measurements of pH were made with a glass electrode. Alkali number measurements were made by the method of Schoch and Jensen (19). The values are reproducible to ±0.3. Nitrogen analyses were made by the micro-Kjeldahl procedure, and phosphorus analyses by a modification of the method of Truog and Meyer (20).

DISCUSSION

Viscosity and Solubility of Dextran—Our results show that the maximum viscosity of culture media of *Leuconostoc mesenteroides* NRRL B-512 does not give an accurate indication of the yield or viscosity of the pure dextran after its isolation. Dextrans with relative viscosities of 2.003 to 2.253 have been isolated in yields of 24 to 25 per cent from unbuffered culture media having viscosities of 73, 202, and 446 centipoises. These differences in viscosity of the culture media appear to be related to the age of the inoculum used. The purified dextrans showed no significant difference in any other of the observed properties. From a calcium carbonate-buffered culture medium with a viscosity of 847 centipoises, dextran having a relative viscosity of 1.719 was isolated in 24 per cent yield. The very high viscosities of culture media buffered with calcium carbonate do not appear to be due to the effect of calcium ions on dextran alone, as is indicated by viscosity measurements on purified dextrans in solutions of calcium acetate (see Table II, foot-notes).

The viscosities of the purified water-soluble dextrans do not correlate with their nitrogen contents. The slight differences in nitrogen content are believed to reflect variation in the efficiency of separation of bacteria from the dextran. Likewise, the solubilities of our dextrans are not related to their nitrogen contents, for the purified water-insoluble dextran from *Leuconostoc mesenteroides* NRRL B-523 has a nitrogen content as low as the water-soluble dextran from *Leuconostoc mesenteroides* NRRL B-512. It is inconceivable that the methods employed for the isolation and purification could have contributed to the solubility of the water-soluble dextran. The solubility and viscosity of these dextrans apparently are inherent properties of the polysaccharides rather than related to combinations of the dextran with protein, as postulated by Stacey (21–23).

Causes and Effects of Decrease in Viscosity of Culture Media—The decrease which occurred in the viscosity of culture media after the formation of dextran was complete appears to be caused mainly by autolysis. The change was almost, but not completely, stopped by heat sterilization of the culture media. This viscosity change was not dependent on the pH or on aeration in unbuffered media. Aeration of buffered media prevents it.

It is not yet known whether "autolysis" results in some selective structural change in dextran, or merely produces a random decrease in molecular size.

Stability of Dextran to Alkali—The alkali numbers in Table II show that Dextrans A, B, and D are not attacked when heated with 0.1 N sodium hydroxide solution, and that Dextrans C and E are only slightly attacked. From other reactions previously reported (2, 3, 9), evidence is available which also indicates the lack of reducing power in dextran. According to the interpretation of alkali numbers (19, 24), our preparations of dextran have very few, if any, reducing groups.

From this viewpoint it is interesting to consider possible modes of termination of dextran molecules which would result in the stability of dextran to alkali. An unterminated cyclic structure, such as is found in the Schardinger dextrins, would be stable to alkali (13), but a cyclic structure for the molecule as a whole would not be in accordance with the pronounced filiform characteristics of water-soluble dextran (8, 17). A chain structure terminated by a unit of glucose-1-phosphate (25), of fructose combined as in sucrose (19), of a hexahydric alcohol such as mannitol, or of gluconic acid (19, 24) would be stable to alkali. Glucose-1-phosphate has been reported to be formed from sucrose by *Leuconostoc mesenteroides* (26), but there is no evidence that it takes part in the synthesis of dextran (27, 28). The possibility that sucrose may be converted directly into dextran without formation of a simpler intermediate (28) could result in the dextran molecule having a terminal fructose unit. No additional evidence can be cited for terminal glucose units in an oxidized or reduced state.

SUMMARY

1. A method has been established for the preparation of water-soluble dextran products of uniformly high viscosities and of high purity from cultures of *Leuconostoc mesenteroides* NRRL B-512 on an unbuffered, unaerated, sucrose medium. This method was dependent on the correlations that the development of maximum viscosity in the culture medium coincided with the end of dextran formation and that thereafter the viscosity of the culture medium and of the dextran decreased.
2. Purified dextrans which were isolated from culture media at their maximum viscosity were characterized by high viscosities; purified dextrans isolated from culture media after their maximum viscosity had been passed had lower viscosities.
3. Accompanying the changes in viscosity of the culture medium, the pH decreased from an initial value of about 7.0, through about 4.6 at the time of maximum viscosity, to a steady value of 3.7 when the viscosity had become very low.
4. Modification of the selected cultural conditions by aeration of the medium or by buffering with calcium carbonate, either singly or in combination, gave no increase in the yield or viscosity of dextran.
5. The solubilities of the water-soluble dextran from *Leuconostoc mesenteroides* NRRL B-512 and of the water-insoluble dextran from *Leuconostoc mesenteroides* NRRL B-523 appear to be inherent properties of the polysaccharides and are not related to their nitrogen contents.
6. In addition to dextran, levan was produced in relatively small amounts in cultures of the strain NRRL B-512.

The use of trade names in this paper does not necessarily constitute endorsement of these products or of the manufacturers thereof.

Grateful acknowledgment is made of the generous assistance and advice of Dr. Robert G. Benedict of the Fermentation Division, who isolated *Leuconostoc mesenteroides* NRRL B-512 from syrupy root beer, and who prepared many of the inocula for our experimental work. The authors wish to express their appreciation to Dr. William C. Haynes for the preparation of some inocula, to Mr. Cecil H. Van Etten and Mrs. Mary B. Wiele of the Analytical and Physical Chemical Division for the nitrogen and phosphorus analyses, and to Mr. C. S. Wise for the alkali number measurements.

BIBLIOGRAPHY

1. Tarr, H. L. A., and Hibbert, H., *Canad. J. Res.*, **5**, 414 (1931).
2. Hassid, W. Z., and Barker, H. A., *J. Biol. Chem.*, **134**, 163 (1940).
3. Sugg, J. Y., and Hehre, E. J., *J. Immunol.*, **43**, 119 (1942).
4. Evans, T. H., Hawkins, W. L., and Hibbert, H., *J. Exp. Med.*, **74**, 511 (1941).
5. Evans, T. H., and Hibbert, H., in Pigman, W. W., and Wolfrom, M. L., Advances in carbohydrate chemistry, New York, **2**, 203 (1946).
6. Stacey, M., *Nature*, **149**, 639 (1942).
7. Hehre, E. J., and Sugg, J. Y., *J. Exp. Med.*, **75**, 339 (1942).
8. Grönwall, A., and Ingelman, B., *Acta physiol. Scand.*, **7**, 97 (1944).
9. Fowler, F. L., Buckland, I. K., Brauns, F., and Hibbert, H., *Canad. J. Res., Sect. B*, **15**, 486 (1937).
10. Hehre, E. J., *J. Biol. Chem.*, **163**, 221 (1946).
11. Stahly, G. L., and Carlson, W. W., U. S. patent 2,203,702 (1940).
12. Owen, W. L., Jr., and Owen, W. L., U. S. patent 2,392,258 (1946).
13. Wilson, E. J., Jr., Schoch, T. J., and Hudson, C. S., *J. Am. Chem. Soc.*, **65**, 1380 (1943).
14. Niven, C. F., Jr., Smiley, K. L., and Sherman, J. M., *J. Biol. Chem.*, **140**, 105 (1941).
15. MacLeod, R. A., and Snell, E. E., *J. Biol. Chem.*, **170**, 351 (1947).
16. Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, **156**, 703 (1944).
17. Jeanes, A., Schieltz, N. C., and Wilham, C. A., *J. Biol. Chem.*, **176**, 617 (1948).
18. Dehn, W. M., Jackson, K. E., and Ballard, D. A., *Ind. and Eng. Chem., Anal. Ed.*, **4**, 413 (1932).
19. Schoch, T. J., and Jensen, C. C., *Ind. and Eng. Chem., Anal. Ed.*, **12**, 531 (1940).
20. Truog, E., and Meyer, A. H., *Ind. and Eng. Chem., Anal. Ed.*, **1**, 136 (1929).
21. Stacey, M., and Youd, F. R., *Biochem. J.*, **32**, 1943 (1938).
22. Stacey, M., *Chem. and Ind.*, **62**, 110 (1943).
23. Stacey, M., in Pigman, W. W., and Wolfrom, M. L., Advances in carbohydrate chemistry, New York, **2**, 163, 185 (1946).
24. Schoch, T. J., Wilson, E. J., Jr., and Hudson, C. S., *J. Am. Chem. Soc.*, **64**, 2871 (1942).
25. Wolfrom, M. L., and Pletcher, D. E., *J. Am. Chem. Soc.*, **63**, 1050 (1941).
26. Kagan, B. O., Latker, S. N., and Zfasman, E. M., *Biokhimiya*, **7**, 93 (1942).
27. Hehre, E. J., *Proc. Soc. Exp. Biol. and Med.*, **54**, 240 (1943).
28. Leibowitz, J., and Hestrin, S., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **5**, 112, 116 (1945).

MOLECULAR ASSOCIATION IN DEXTRAN AND IN BRANCHED AMYLACEOUS CARBOHYDRATES

By ALLENE JEANES, N. C. SCHIELTZ,* and C. A. WILHAM

(*From the Starch and Dextrose Division and the Analytical and Physical Chemical Division, Northern Regional Research Laboratory,*† *Peoria, Illinois*)

Plate 3

(Received for publication, June 21, 1948)

A property of polysaccharides which is of major importance both practically and fundamentally is orderly association between portions of the same or of adjacent molecules. This property depends upon both the physical and chemical constitution of the polysaccharide and data on it provide a means for correlating physical properties with chemical structure. Little specific information is available on molecular association in branched polysaccharides. The industrial importance of starch and the biological significance of glycogen and certain bacterial polysaccharides make such information desirable.

This paper presents information on molecular association in the bacterial polysaccharide, dextran from *Leuconostoc mesenteroides*, and in branched amylaceous substances. Orderly molecular association has been detected by x-ray analysis, and the tendency towards orderly association has been related to the physical and chemical constitution of these polysaccharides. Data are given on typical x-ray diffraction powder patterns of dextran, and on the relation of molecular association to the hydration and ease of dissolution of branched polysaccharides.

Chemical and Physical Nature of Dextran—Dextran is known to be a polymer of α-D-glucopyranose (1). Methylation studies on a water-soluble dextran (2) have shown that the molecule has a branched structure in which the predominant glucosidic linkage is 1,6- while 1,4-linkages occur at the points of branching. A point of branching for every 5 glucose units was indicated by these data. Less quantitatively exact methylation studies on other dextran preparations have indicated fewer side chains (1, 3). In amylaceous substances the predominant glucosidic linkage is α-1,4- while 1,6-linkages occur at the points of branching (4). The presence of 1,6-linkages in starch makes further knowledge of other polysaccharides containing this linkage desirable for comparative purposes.

---

* Present address, United States Department of the Interior, Bureau of Reclamation, Denver, Colorado.

† One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

Dextran has been shown to be filiform in nature by electron microscopy (5), by ultracentrifugation (6), and by birefringence of flow (6). Grönwall and Ingelman (6) reported that the molecular weight of the dextran studied by them was of the order of "many millions," but accurate evaluation was prevented by the inhomogeneity of size of the molecules.

Dextran molecules appear to differ in size (7) and probably in some details of chemical structure when prepared under different conditions and by different strains of *Leuconostoc mesenteroides*. Our observations on the water-soluble dextran from *Leuconostoc mesenteroides* NRRL B-512 which was used in this study indicate that, with the possible exception of the degree of branching, this dextran appears to conform to the structural characteristics indicated above. It cannot be assumed that the methylation data available (2) apply exactly to our dextran.

Molecular Order and Association—Samples of polysaccharides which produce x-ray diffraction powder patterns containing a set of diffraction lines are called "crystalline." A crystalline polysaccharide is ordinarily a mixture of regions which range in degree of molecular order between the theoretical extremes of complete crystallinity and complete randomness. A single polysaccharide molecule may extend through several different regions (8). In the crystalline regions segments of primary valence chains are in ordered spatial arrangement where energy relations favor systematic lateral association between chains through hydrogen bonds. In amorphous regions chain segments are in disorderly arrangement. X-ray patterns from ordinary crystalline polysaccharides are composites of the diffraction lines which originate in the crystalline regions and of the halo of diffuse reflections from the amorphous regions. Amorphous polysaccharides, which produce only diffuse x-ray reflections, are believed to vary in the extent and in the degree of randomness of molecular association.

EXPERIMENTAL

Materials and Methods—Unless otherwise stated, the dextran used has been the water-soluble product from *Leuconostoc mesenteroides* NRRL B-512 and was prepared and characterized as described in a previous study (7).

Humidification in an atmosphere having 100 per cent relative humidity at 25° has been used to convert amorphous samples of glutinous cornstarch, amylose-free corn amylopectin, and certain fractions from acid-hydrolyzed dextran to the crystalline state. Dextrans and acid-hydrolyzed dextrans have produced line patterns after treatment under special conditions with specific optimum concentrations of aqueous ethanol. Crystallization has also been found to develop in dextran when its aqueous pastes were dried at elevated temperatures. These general methods, or the fundamental principles underlying them, have been used previously in studies on starch (9, 10) or other polysaccharides (11).

X-ray patterns were made with unfiltered copper radiation transmitted through the finely pulverized samples which were packed in holders 1 mm. thick. Before the pattern was taken, all samples which had been stored several days in an atmosphere having 100 per cent relative humidity at 25° were permitted to dry in the air from 1 to several hours to reach a satisfactory condition for mounting. The exact state of hydration of samples when x-rayed has not been defined.

Amorphous Dextran—Dextran is normally isolated as a fluffy white solid (7) which gives an amorphous x-ray pattern, and in which the randomness of molecular association is very high. Unless otherwise stated, amorphous dextran has been the starting material used in all the experimental work described here.

Amorphous dextran takes up 12 to 13 per cent moisture when equilibrated in an atmosphere having 50 per cent relative humidity at 25°. When a small amount of amorphous dextran was exposed in an atmosphere having 100 per cent relative humidity at 25°, it changed to a clear, thick syrup within 2 to 3 hours. After 3 days under these conditions, x-ray examination showed the dextran still to be amorphous. These observations have been made on dextrans with relative viscosity[1] of 2.003, 1.816, and 1.414.

X-ray Data and Patterns—Three general types of x-ray diffraction powder patterns, one of which has two modifications, have been obtained from dextran and acid-hydrolyzed dextran. The x-ray data for these typical patterns are given in Table I and illustrations are given in Fig. 1.

Preparation and Properties of Samples

Dextran Treated with Aqueous Ethanol—0.5 gm. samples of dextran (relative viscosity[1] 1.816) were dissolved in 15 cc. of distilled water in small glass-stoppered flasks, and absolute ethanol was added to make ethanol concentrations of 40, 50, 60, 70, and 80 per cent by volume, respectively. The dextran precipitated and was allowed to stand under the aqueous ethanol for 7 days at 25°. The precipitates were then removed and dried *in vacuo* over anhydrous calcium chloride at 25°. When dry, the sample from 60 per cent ethanol pulverized with great ease and the sample from 70 per cent ethanol pulverized easily, but all the other samples could be broken only into lumps. The samples from 40 and 50 per cent ethanol produced amorphous x-ray patterns, those from 60 and 70 per cent ethanol produced line patterns of type L-1, and the sample from 80 per cent ethanol gave a pattern with traces of lines on a diffuse background.

Humidification for 3 days in an atmosphere saturated with water vapor at 25° produced no apparent change in the dry, pulverized samples from 60 and 70 per cent ethanol, but resulted in increased sharpness of the type

---

[1] Viscosities were measured on 0.5 per cent solutions in water at 25° (7).

L-1 patterns produced. When humidified, the sample from 40 per cent ethanol dissolved readily to a clear syrup, which, after drying, produced an amorphous pattern. Similarly, the samples from 50 and 80 per cent ethanol formed cloudy syrups, which, after drying, produced amorphous x-ray patterns showing traces of lines.

Extension of the time of treatment in aqueous ethanol to 35 days resulted in an amorphous product from 40 per cent ethanol and a crystalline product from 50 per cent ethanol.

TABLE I

Interplanar Spacings and Estimated Intensities of Typical Dextran Patterns

Type L-1		Type L-2		Type L-3		Type L-3′	
A		*A*		*A*		*A*	
13.0	Weak					13.0	Very weak
6.50	"			6.93	Medium	7.80	" "
5.76	Medium	5.65	Very weak	6.13	"	6.02	Medium
5.05	"	4.95	Medium	5.01	"	5.05	Weak
4.69	"	4.52	"	4.52	Very strong	4.55	Strong
4.32	Medium strong	4.00	Very, very weak			4.25	Very, very weak
3.90	Very weak			3.89	Strong	3.90	Medium
3.56	" "	3.57	Very weak	3.49	Medium	3.47	"
3.33	Medium	3.24	Weak				
3.04	"	3.06	Very, very weak	3.11	Very weak		
2.79	Very, very weak			3.00	Weak	3.04	Medium
2.65	" "	2.55	Very, very weak	2.84	Very weak	2.84	Very, very weak
2.50	" "	2.36	" "	2.71	" "	2.65	" "
2.37	" "			2.59	" "	2.59	" "
2.26	" "			2.43	Weak	2.45	" "
				2.26	"	2.26	" "
				2.18	"	2.19	" "

Essentially the same results were obtained by use of dextrans having a relative viscosity of 2.003 and 1.414. The pattern from the latter dextran is shown in Fig. 1, *a*.

Dextran; Drying of Aqueous Pastes—A viscous paste of dextran (relative viscosity[1] 2.003) was dried in two parts; one at 25°, the other at 115°. The former gave an amorphous pattern, the latter gave a poor quality x-ray line pattern of the type L-2 (see Fig. 1, *b*). A much thinner paste, when dried more slowly at 50°, produced an x-ray pattern of type L-1 of good quality.

Dextran; Products of Acid Hydrolysis—Dextran was hydrolyzed by 0.5

N sulfuric acid at 90° for 1, 2, and 4 hours, respectively, and the fractions of highest molecular weight were isolated from the hydrolysates. Complete details of these hydrolyses and fractionations will be given elsewhere.[2]

The fractions of highest molecular weight were separated from hydrolysis products of smaller size by dialysis of the sulfate-free solution and by repeated precipitation from aqueous solution by the addition of ethanol to make a 60 per cent ethanol concentration. During the fractional precipitations, the products from 2 and 4 hour hydrolyses became incompletely soluble in hot water. After the last fractional precipitation, the subfraction which had become insoluble in hot water was removed and found to give a good quality x-ray line pattern of type L-3. The main fraction was isolated from the clear supernatant in a manner analogous to that described for amorphous dextran (7).

These 60 per cent ethanol-insoluble fractions from 1 and 2 hour hydrolyses gave amorphous x-ray patterns and had $[\alpha]_D^{25} = +199°$ (in 1 N sodium hydroxide, $C = 1$) and about 65 and 40 glucose units, respectively, per reducing group. Ethoxyl analysis showed them to contain no ethanol. X-ray line patterns of the type L-3 and of very good quality were obtained after humidification or after treatment for 2 days with aqueous ethanol. Although humidification, which caused the amorphous samples to turn to cloudy pastes, appeared to make no significant change in the dried, powdery products from the treatment with aqueous ethanol, it caused these samples to produce intensified and sharper x-ray reflections.

Other fractions from 2 and 4 hour hydrolyses, the isolations of which are not described specifically here, have produced strong x-ray patterns, as is shown in Figs. 1 *c* and 1 *d*.

Dextrans Prepared by Other Investigators—X-ray examination of the dextran of Hassid and Barker[3] (1) and of the dextran from *Leuconostoc dextranicum*,[4] which is, presumably, the dextran referred to by Fairhead, Hunter, and Hibbert (13), gave results comparable to those for *Leuconostoc mesenteroides* dextran. In the states in which they were obtained, these samples produced amorphous x-ray patterns, but after treatment with 60 per cent ethanol the products gave x-ray line patterns of type L-1.

The dextran from *Leuconostoc dextranicum* dissolved readily to form aqueous pastes of very low viscosity. The dextran of Hassid and Barker, the dense, horny appearance of which was indicative of close molecular packing and which has been described as water-insoluble (1), was obtained in 2 per cent aqueous solution by vigorous mechanical shaking. After precipitation of the dextran from this solution in the manner described for the preparation of amorphous dextran (7), the fluffy product was much more readily soluble in water than was the original sample.

---

[2] Wilham, C. A., and Jeanes, A., unpublished data.

[3] We are indebted to Professor W. Z. Hassid of the University of California for this sample.

[4] This sample was furnished by Dr. Richard E. Reeves of the Southern Regional Research Laboratory, New Orleans, Louisiana, who obtained it from Professor Harold Hibbert and used it in the investigation reported in (12).

Water-Insoluble Dextran—In the state in which they were isolated, both of the purified fractions of the water-insoluble dextran from *Leuconostoc mesenteroides* NRRL B-523 (7) produced essentially amorphous x-ray patterns. Weak interferences were produced at 9.5 and 4.25 A by the fraction which was insoluble in the culture medium, and at 20.0 and 9.5 A by the fraction which was originally soluble in the culture medium but which became insoluble during purification. After exposure in 100 per cent relative humidity for 3 days at 25°, the first mentioned fraction produced a weak crystalline pattern which had an interference at 9.5 A, and the third through the ninth interferences of the dextran pattern, type L-3′ (see Table I).

Each of these fractions appeared luminous under crossed Nicol prisms but no extinction was observed.

Amylaceous Carbohydrates—3 days humidification of corn amylose, which had been isolated from the amylose-butanol complex (14) as a chemically reactive, dry powder,[5] changed it to a coherent, elastic mass, and caused its x-ray pattern to change from a V to a B type. (When starch crystallizes in the presence of alcohol, usually a V pattern results; when it crystallizes in the presence of water alone, A or B patterns result.) Humidification of similarly prepared potato amylose resulted, after 3 days, in the replacement of its original well defined V pattern by an amorphous pattern. Continuing humidification for 3 weeks resulted in the development of a poor line pattern. Katz (9) observed that the V pattern of precipitated wheat starch was changed to the B pattern by humidification.

Methanol-extracted (15) glutinous corn-starch, contaminated with about 2 per cent ordinary corn-starch, was prepared in an amorphous state by precipitation of an aqueous paste in ethanol.[5] Humidification for 7 days caused the starch, which originally produced the amorphous x-ray pattern shown in Fig. 1, *e*, to produce the type A pattern shown in Fig. 1, *f*. Native granules of glutinous corn-starch are known to produce an x-ray line pattern (10).

A dry, amorphous sample of corn amylopectin, which had been treated with cotton to remove last traces of amylose (16), produced an A type starch pattern after 3 days humidification.

Glycogen has been reported to give only amorphous patterns under treatments which cause starch to give line patterns (17, 18). Application of the technique of treatment with aqueous ethanol as described for dextran resulted, even after 8 weeks under aqueous ethanol, in amorphous patterns only. Similarly, only amorphous patterns were obtained from the β-amylase limit dextrin of glutinous corn-starch.

---

[5] Jeanes, A., Deane, R. A., Whistler, R. L., and Hilbert, G. E., in preparation.

Humidification caused both glycogen and β-amylase limit dextrin to turn to clear syrups, which, after drying, produced amorphous x-ray patterns.

Observations on Other Physical Properties

The behavior of samples under conditions of 100 per cent relative humidity at 25° not only reveals small differences in their ability to hydrate and dissolve but is also an indication of the x-ray pattern which the sample will give after this treatment. The variation of the humidified samples from rather dry, apparently unchanged powders through opaque to cloudy pastes to clear syrups directly paralleled the decreasing degree of orderly association, as indicated by the x-ray patterns.

Likewise, the order of solubility paralleled the physical condition of the sample. Thus, fluffy, amorphous dextran dissolved readily merely by exposure in an atmosphere saturated with water vapor at 25°, but the pulverized "glassy" product from a dextran paste dried at 115° dissolved slowly in cold water (20°), and the pulverized, "glassy" product from a paste dried slowly at 50° required heating to 70° to dissolve it in water. Fluffy, amorphous samples of acid-hydrolyzed dextrans dissolved readily in cold water, but the powdery crystalline products from the action of 60 per cent ethanol on these fractions as well as the pulverized, "glassy" crystalline products of humidification required heating in water at 100° to give clear solutions.

Formation of Filaments from Dextran—Filaments have been obtained from dextrans having a relative viscosity[1] of 1.855 and 2.003 and from "autolyzed" dextrans having a relative viscosity of 1.414 and 1.565. Aqueous pastes of the proper consistency for filament formation were obtained from "autolyzed" dextrans by 2 days humidification in air saturated with water vapor at 25°, but pastes obtained from the other dextrans under these conditions were too rubbery and viscous and had to be thinned by addition of water. When, under normal atmospheric conditions, a probe was touched to these pastes and then steadily pulled away, a lustrous filament was obtained. A filament many feet in length has been obtained by the use of a small mechanical reel. X-ray examination of a bundle of these fibers gave an amorphous pattern.

Filaments could not be obtained similarly from the dextran of Hassid and Barker[3] nor from glutinous corn-starch.

DISCUSSION

The emphasis that has been given to the amorphous nature of branched amylaceous substances in contrast to the crystalline nature of the linear substances has led recently to the erroneous belief that only the linear molecules can produce x-ray line patterns (19). In general, irregularity in molecular shape or structure is believed to interfere with orderly association (8). For example, the amorphous nature of the unbranched polysaccharide, lichenin, has been attributed to molecular irregularity resulting from the presence of both 1,3- and 1,4-glucosidic linkages (20). In amylaceous substances, branches are generally believed to provide the irregularity that interferes with crystallization. However, the view has been expressed that orderly association occurs between branches of sufficient length (21, 22) but not if the branches are modified or shortened (22). The results reported here on corn amylopectin, glutinous corn-starch, the β-amylase limit dextrin of glutinous corn-starch, and glycogen, in agreement with observations of Schoch and French (22), show that the tendency towards orderly molecular association decreases in the order stated; that is, in the order of increasing number or shortness of side chains (21, 23). However, the difference reported here in the behavior of potato as compared with corn amylose indicates that some still unrecognized factor can interfere with crystallization even in linear molecules which are apparently uniform in structure.

From the extremely careful methylation study of Levi, Hawkins, and Hibbert on a water-soluble dextran from *Leuconostoc mesenteroides* (2), it appears that this substance has greater structural irregularity and more numerous as well as shorter branches than glycogen. In view of this and of the influential rôle that branching seems to play in retarding crystallization in amylaceous polysaccharides, the observation of crystallinity in dextran seems to be anomalous. It is possible that crystallization might be more favored in dextran than in glycogen by the predominance of glucosidic linkages involving a primary position on carbon 6. It is also possible that the concept of the structure of dextran is inaccurate and that clarification may have to await further structural information on dextrans of specific origin.

Dextran produces a series of typical x-ray diffraction powder patterns which are entirely different from the patterns of starch. The relative intensities of the lines and the background in patterns from the high molecular weight dextrans indicate that the amount of crystalline material present is rather small. A treatment which resulted in crystallization of the high molecular weight dextran from *Leuconostoc mesenteroides* NRRL B-512 also resulted in about the same degree of crystallinity in two other dextrans from widely different sources. This is interpreted to indicate that the x-ray line pattern common to these three dextrans is due to a structure which is characteristic of dextran rather than to the presence of a small fraction of material with exceptional crystallizing ability.

The fractions of highest molecular weight from 1, 2, and 4 hour acid hydrolyses of dextran progressively developed crystallinity more readily and completely than dextran. This might result from random reduction of molecular size or from increased regularity in size or structure of the molecules during acid hydrolysis. Increased regularity in structure is to be expected from preferential hydrolysis of the α-1,4-glucosidic linkages, which we have found to have a critical increment 3000 calories per mole less than that of α-1,6-linkages.[2]

Numerous water-insoluble polysaccharides such as amylose (14), cellulose (19), pectins (24), and some of the fractions produced by *Leuconostoc mesenteroides* NRRL B-512 (7), or obtained from its water-soluble dextran by acid hydrolysis, produce strong x-ray line patterns. Unlike all these other substances, the dextran from *Leuconostoc mesenteroides* NRRL B-523 gave an amorphous x-ray diffraction pattern, was not horny, and yet was water-insoluble. Molecular association in this dextran must be extensive but arrangement of the associated chain segments in a 3 dimensional periodic pattern is lacking.

Investigations on cellulose esters (25) and proteins (26) have established that water, or water and heat, facilitate motion and crystallization of chain segments of these substances in the solid state. Similar observations have been made on starch granules (27) and on starch coacervates (22). Our observations provide further examples of this principle. Exposure of solid samples in an atmosphere saturated with water vapor at 25° caused some amorphous substances to assume a crystalline state, crystalline substances to change from one modification to another or develop sharper patterns, and one substance passed through an amorphous state during its transition from one crystalline state to another. Crystallization resulted when a dextran paste was warmed while being dried slowly, but did not result when the slow drying was at 25°. Hydrated masses of dextran developed crystallinity when allowed to stand under specific optimum concentrations of aqueous ethanol, presumably because a critical state of dehydration was obtained which was favorable to molecular orientation and association.

SUMMARY

1. Orderly molecular association has been demonstrated by x-ray analysis in branched chain polysaccharides including water-soluble dextran from *Leuconostoc mesenteroides* NRRL B-512, corn amylopectin, and glu-

tinous corn-starch. Under the same conditions, glycogen and the β-amylase limit dextrin from glutinous corn-starch did not develop detectable molecular organization.

2. In view of the fact that amylaceous substances which have short branches do not develop orderly molecular association, the crystallization of water-soluble dextran was not to be expected on the basis of the generally held concept that dextran has numerous, short branches.

3. With an increase in the degree of orderly molecular association, as detected by x-ray analysis, there was a decrease in the ability of water-soluble dextran, acid-hydrolyzed dextran, and amylaceous substances to dissolve in water. A more sensitive test of the ability of these substances to hydrate was their behavior under conditions of 100 per cent relative humidity at 25°.

4. Data for a series of typical x-ray diffraction powder patterns have been obtained and used to characterize not only dextrans from three different sources but also acid-hydrolyzed dextrans.

5. Filaments many feet in length have been spun from aqueous pastes of dextran from *Leuconostoc mesenteroides* NRRL B-512.

Grateful acknowledgment is made of the helpful criticisms of Mr. C. E. Rist and Dr. N. N. Hellman.

BIBLIOGRAPHY

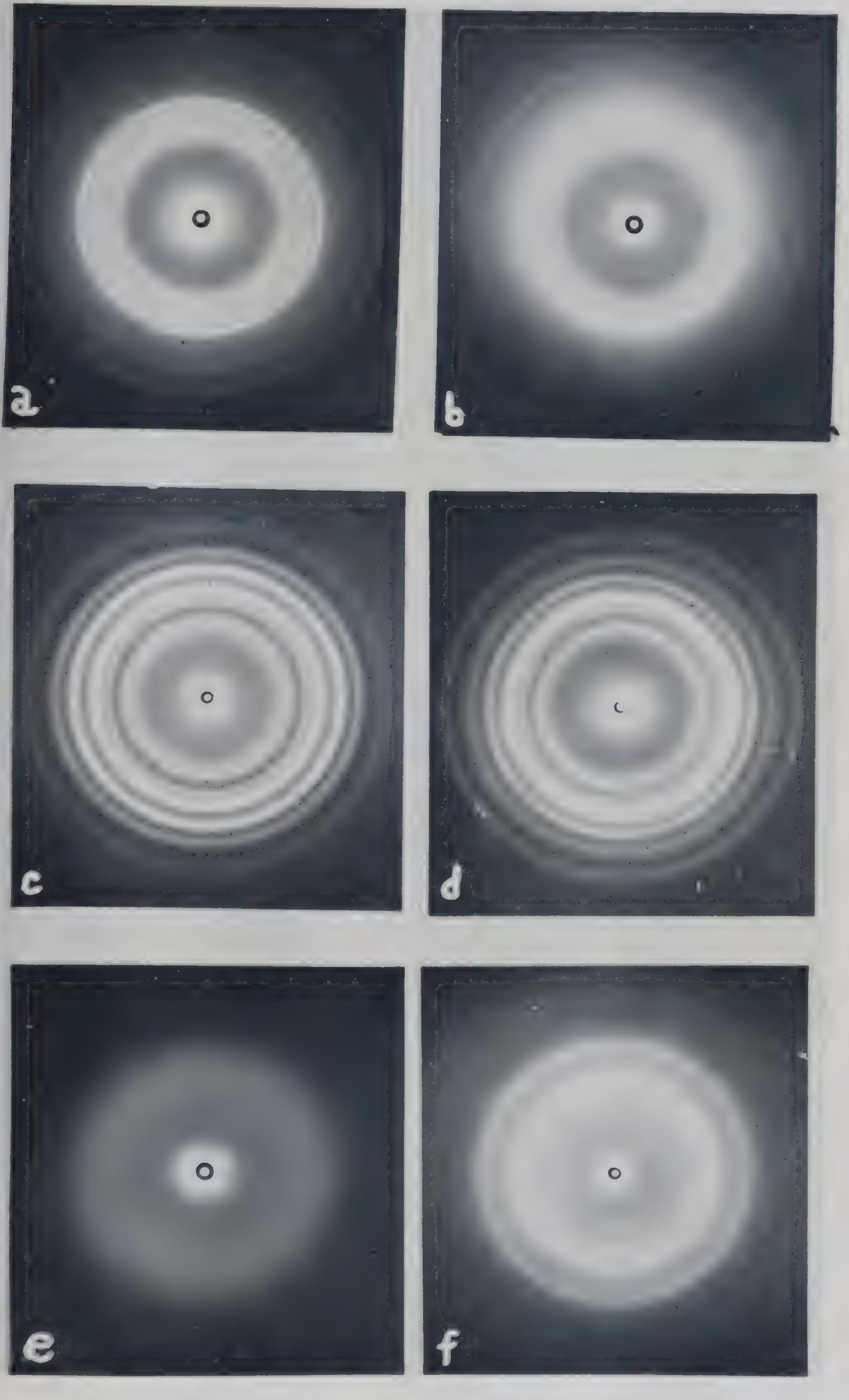
1. Hassid, W. Z., and Barker, H. A., *J. Biol. Chem.*, **134**, 163 (1940).
2. Levi, I., Hawkins, W. L., and Hibbert, H., *J. Am. Chem. Soc.*, **64**, 1959 (1942).
3. Peat, S., Schlüchterer, E., and Stacey, M., *J. Chem. Soc.*, 581 (1939).
4. Barker, C. C., Hirst, E. L., and Young, G. T., *Nature*, **147**, 296 (1941). Montgomery, E. M., Weakley, F. B., and Hilbert, G. E., *J. Am. Chem. Soc.*, **69**, 2249 (1947).
5. Ingelman, B., and Siegbahn, K., *Nature*, **154**, 237 (1944); *Ark. Kemi, Mineral. Geol.*, **18 B**, 1 (1944).
6. Grönwall, A., and Ingelman, B., *Acta physiol. Scand.*, **7**, 97 (1944).
7. Jeanes, A., Wilham, C. A., and Miers, J. C., *J. Biol. Chem.*, **176**, 603 (1948).
8. Alfrey, T., Jr., Mechanical behavior of high polymers, New York, 340 (1948).
9. Katz, J. R., *Z. phys. Chem., Abt. A*, **170**, 430 (1934).
10. Bear, R. S., and French, D., *J. Am. Chem. Soc.*, **63**, 2298 (1941).
11. Berner, E., *Ber. chem. Ges.*, **64**, 842 (1931).
12. Reeves, R. E., *J. Biol. Chem.*, **154**, 49 (1944).
13. Fairhead, E. C., Hunter, M. J., and Hibbert, H., *Canad. J. Res., Sect. B*, **16**, 151 (1938).
14. Schoch, T. J., *J. Am. Chem. Soc.*, **64**, 2957 (1942).
15. Schoch, T. J., *J. Am. Chem. Soc.*, **64**, 2954 (1942).
16. Pacsu, E., and Mullen, J. W., 2nd, *J. Am. Chem. Soc.*, **63**, 1168 (1941).
17. Samec, M., Katz, J. R., and Derksen, J. C., *Z. phys. Chem., Abt. A*, **158**, 321 (1932).
18. Bear, R. S., and Cori, C. F., *J. Biol. Chem.*, **140**, 111 (1941).
19. Peat, S., in Annual review of biochemistry, Stanford University, **15**, 78 (1946).

20. Meyer, K. H., and Gürtler, P., *Helv. chim. acta*, **30,** 751 (1947).
21. Meyer, K. H., and Bernfeld, P., *Helv. chim. acta*, **23,** 875 (1940). Meyer, K. H., and Fuld, M., *Helv. chim. acta*, **24,** 375 (1941).
22. Schoch, T. J., and French, D., *Cereal Chem.*, **24,** 231 (1947).
23. Haworth, W. N., Kitchen, H., and Peat, S., *J. Chem. Soc.*, 619 (1943).
24. Speiser, R., Copley, M. J., and Nutting, G. C., *J. Phys. and Colloid Chem.*, **51,** 117 (1947).
25. Baker, W. O., Fuller, C. S., and Pape, N. R., *J. Am. Chem. Soc.*, **64,** 776 (1942).
26. Senti, F. R., Copley, M. J., and Nutting, G. C., *J. Phys. Chem.*, **49,** 192 (1945).
27. Sair, L., and Fetzer, W. R., *Ind. and Eng. Chem.*, **36,** 205 (1944).

EXPLANATION OF PLATE

Plate 3

Fig. 1. X-ray diffraction patterns of dextran, acid-hydrolyzed dextrans, and glutinous corn-starch. *a*, type L-1 from dextran of relative viscosity 1.414 after treatment with 60 per cent ethanol followed by humidification; *b*, type L-2 from a dextran paste dried at 115°; *c*, dextran type L-3 from a fraction of 4 hour acid hydrolysis after treatment with 70 per cent ethanol followed by humidification; *d*, dextran type L-3′ from a fraction of 2 hour acid hydrolysis after humidification; *e*, pattern from amorphous glutinous corn-starch; *f*, type A starch pattern from amorphous glutinous corn-starch after humidification.



Jeanes, Schieltz, and Wilham: Dextran and amylaceous carbohydrates

FUNCTION OF THE VITAMIN B_6 GROUP: MECHANISM OF TRANSAMINATION

By W. W. UMBREIT,* D. J. O'KANE,† and I. C. GUNSALUS‡

(From the Laboratory of Bacteriology, College of Agriculture, Cornell University, Ithaca)

(Received for publication, June 24, 1948)

The rôle of vitamin B_6 in amino acid metabolism has been well established through enzyme studies (1–3) and by the substitution for various amino acids in growth studies (4–6).

The coenzyme form, synthetic pyridoxal phosphate (7), functions as the coenzyme for amino acid decarboxylases (8–10), transaminases (2, 3), tryptophan formation (11), and tryptophan breakdown by tryptophanase (12). The reactions involved in the amino acid replacement of vitamin B_6 have not been determined.

The mechanism by which pyridoxal phosphate functions is still in question. Snell (13), however, found that pyridoxal and pyridoxamine were readily and reversibly interconverted by *in vitro* transamination and suggested on this basis that biological transamination might be mediated by the transfer of the amino group via the aldehyde and amino forms of vitamin B_6 to the keto acid. The demonstration of pyridoxal phosphate as the coenzyme of transamination quite naturally suggested the existence of pyridoxamine phosphate and its possible rôle in those systems for which pyridoxal phosphate is the coenzyme.

Umbreit, O'Kane, and Gunsalus (14) found activation of the apotransaminase of dried bacteria by "pyridoxamine phosphate" which had been prepared by the method of Snell for the interconversion of pyridoxal and pyridoxamine (13). The pyridoxamine phosphate was judged to be free of pyridoxal phosphate by its lack of coenzyme activity for tyrosine apodecarboxylase and by the spectrum. Later Ames, Sarma, and Elvehjem (15) found pyridoxamine phosphate, prepared as above to stimulate the transaminase in liver homogenates from vitamin B_6-deficient animals. These data could be interpreted as substantiating the hypothesis of pyridoxamine phosphate as an intermediate in transamination, although the alternative possibility of the transformation of the pyridoxamine phosphate to pyridoxal phosphate by a separate enzyme was not excluded. The latter type of transformation occurs in *Streptococcus faecalis*, as indicated by the function of pyridoxamine for tyrosine apodecarboxylase only after incubation with cells and pyruvate (16).

---

* Present address, Merck Institute for Therapeutic Research, Rahway, New Jersey.

† Present address, University of Pennsylvania.

‡ Present address, Indiana University.

A critical test of the mechanism of transamination and the possible rôle of the two coenzyme forms as intermediates would require a purified apoenzyme in which the multiplicity of side reactions, possible in dried bacterial cells or homogenates, was eliminated. For this purpose the glutamic-aspartic transaminase of pig heart was resolved and purified (17). On test the pyridoxamine phosphate preparations were not active, whereas the pyridoxal phosphate activated the enzyme. The pyridoxamine phosphate was, as mentioned previously, also inactive for tyrosine apodecarboxylase and for tryptophanase. In addition to the enzyme data, the spectra of pyridoxal and pyridoxamine and their respective phosphates, by which all four may be differentiated, are reported. Recently, Snell *et al.* have reported differential analysis of the four by growth assays (18, 19).

Methods

Enzyme—The enzymes were prepared as previously reported from this laboratory: the tyrosine decarboxylase from cells grown in vitamin B_6-deficient medium and dried *in vacuo* (20); the bacterial apotransaminase by similar process, except that the cells were grown in a neutral medium (2); the purified glutamic-aspartic apotransaminase by resolution of the enzyme from pig heart (17) and its purification by the method of Green (21).

Coenzymes—Synthetic barium pyridoxal phosphate (7) was used. Pyridoxamine phosphate was not synthesized directly, but was prepared by treating pyridoxal phosphate with glutamic acid and heat according to the method of Snell (13) for the transformation of pyridoxal to pyridoxamine. No inorganic phosphate was released by the heating and pyridoxal phosphate was absent as shown by the enzymatic test with tyrosine apodecarboxylase and by the spectrum, as indicated below. The reaction could also be reversed, although not completely, by treating the pyridoxamine phosphate samples with α-ketoglutarate, again according to Snell's method. The preparation of pyridoxamine phosphate in pure form free from pyridoxal phosphate and its chemical characterization is essential for the proof of mechanism of the reactions studied.

Spectra of Pyridoxal, Pyridoxamine, and Their Phosphates

Pyridoxal, in contrast to other members of the vitamin B_6 group, exhibits a yellow color at alkaline reaction with an absorption maximum at 385 mμ. This should not be confused with the ultraviolet spectrum which is possessed by all members of the group. The intensity of the absorption is increased in pyridoxal phosphate, as compared to pyridoxal, without appreciable shift in the wave-length of the band. The spectra for these two compounds at various pH levels are shown in Fig. 1. Especially to be noted is the absorption at pH 7; *i.e.*, the pyridoxal absorption is nil, whereas the pyridoxal phosphate absorption is more than 90 per cent maximum.

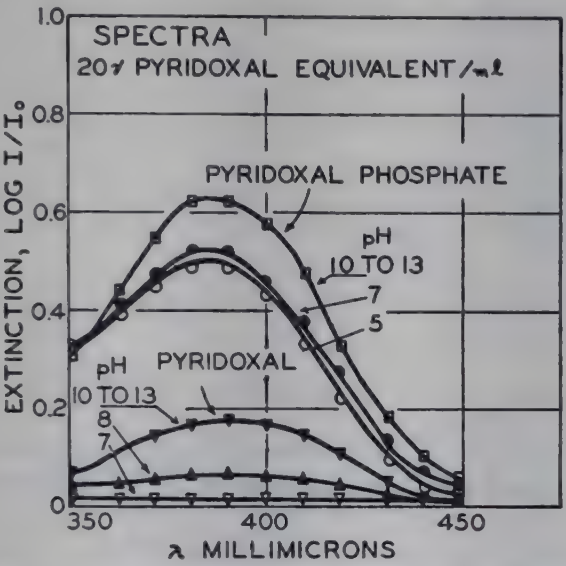


FIG. 1. Effect of pH on absorption spectra of pyridoxal and pyridoxal phosphate

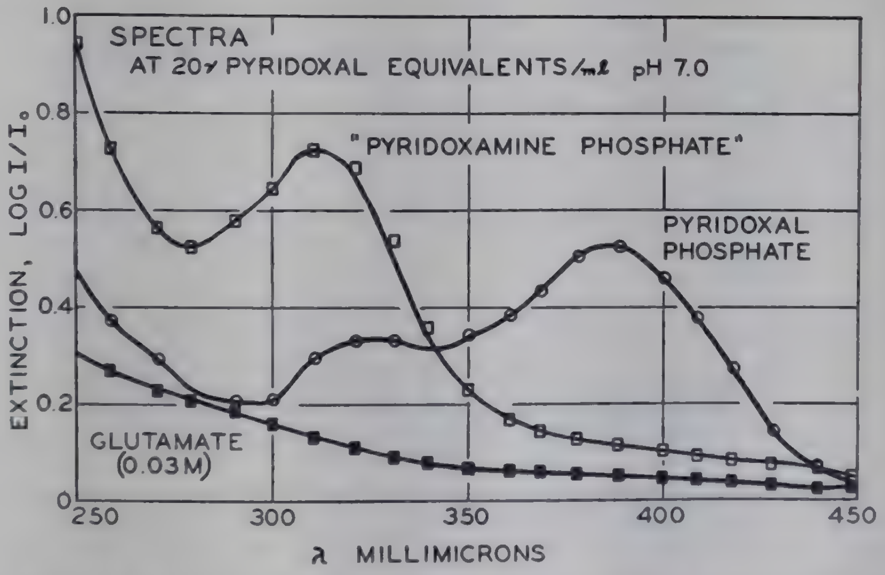


FIG. 2. Spectra of pyridoxal phosphate and pyridoxamine phosphate. Pyridoxamine phosphate obtained by heating pyridoxal phosphate (100 γ per ml.) at 121° for 30 minutes with 0.03 M glutamate, pH 7.0.

If one heats pyridoxal phosphate with glutamic acid (30 minutes, 121°), the absorption at 385 mμ disappears with the formation of pyridoxamine phosphate. The spectra of these compounds, in the range 250 to 450 mμ at pH 7, are shown in Fig. 2. Incubation of pyridoxal phosphate with glutamic acid or with glutamic-aspartic transaminase results in a slight decrease in absorption at 385 mμ and a shift of the maximum toward the longer wave-lengths. This may possibly indicate a coordination of the amino groups with the free aldehyde of pyridoxal.

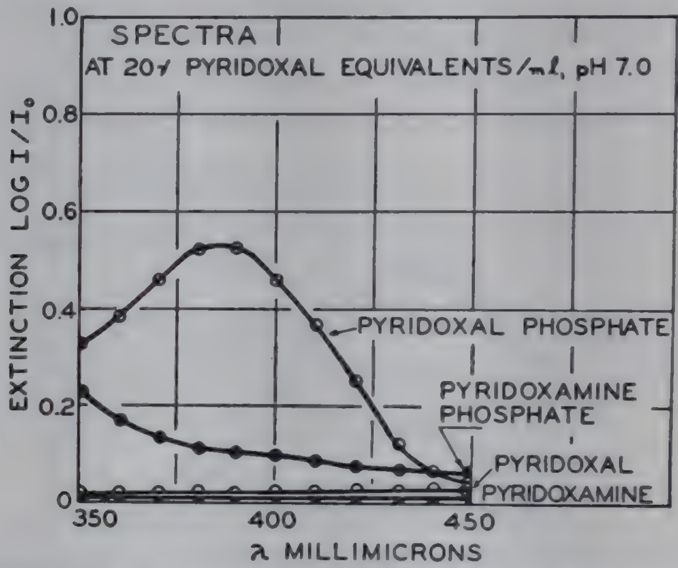


FIG. 3. Differentiation of pyridoxal and pyridoxamine from their phosphates by spectrum.

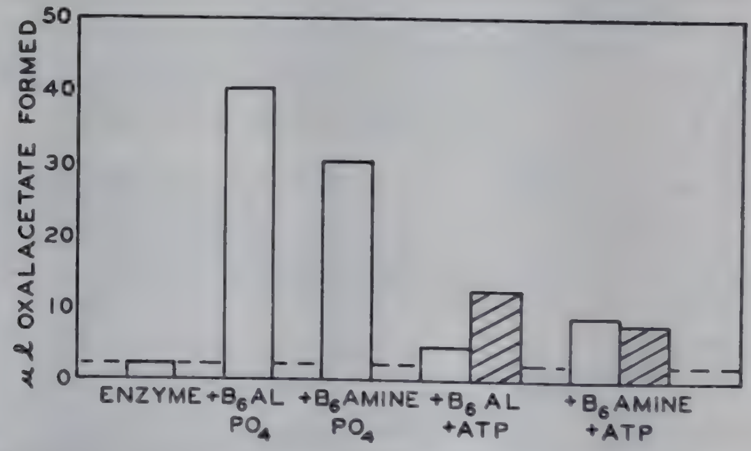


FIG. 4. Activation of apotransaminase of vitamin B_6-deficient *Streptococcus faecalis*.

The absorption spectra for free pyridoxal and pyridoxamine and their phosphates at pH 7 are shown in Fig. 3. Thus the pyridoxal and pyridoxamine phosphates can be distinguished, and each distinguished from its non-phosphorylated analogue. Pyridoxal may be differentiated from pyridoxamine by the increased absorption at alkaline reaction (Fig. 1).

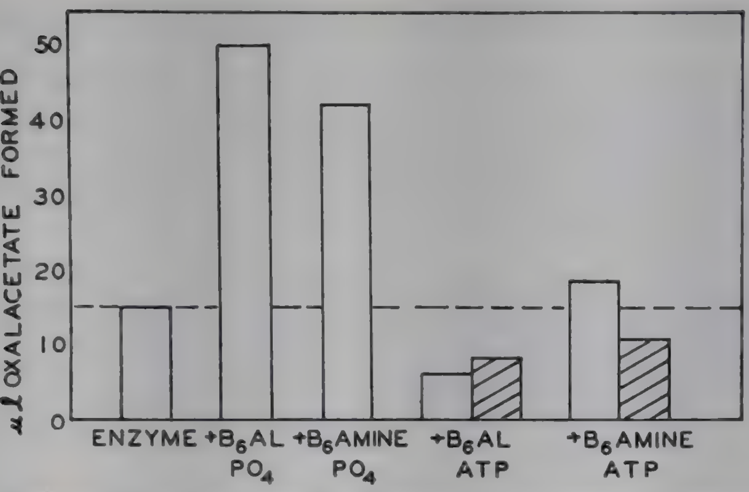


FIG. 5. Activation of cell-free resolved bacterial apotransaminase with pyridoxal and pyridoxamine phosphate.

TABLE I

Pyridoxal Phosphate and Pyridoxamine Phosphate Activation of Transaminase Streptococcus faecalis R, Dried Cells

Per Warburg cup, 0.5 ml. of 0.2 M phosphate buffer, pH 6.5, enzyme, coenzyme, water to 1.9 ml.; 1st side arm, 0.5 ml. of 0.4 M aspartic acid, 0.3 ml. of 0.2 M α-ketoglutaric acid; 2nd side arm, 0.5 ml. of aniline citrate.

Sample, treatment	Microliters oxalacetate formed per 30 min.				
	None	Pyridoxal phosphate	Pyridoxamine phosphate*	Pyridoxal ATP	Pyridoxamine ATP
		per 10 γ	*per 10 γ*	*per 10 γ per 1 mg.*	*per 10 γ per 1 mg.*
T_4, autolyzed 18 hrs., 37° 0.1 M phosphate, pH 7.5, supernatant dialyzed	14	50	41	7	10
R_6T, autolyzed 18 hrs., 37°, 0.1 M phosphate, pH 7.3, supernatant used immediately	14	58	73	16	19
Run†					
T_4, 7 days	18	61	35		
T_5	2	37	34		
R_6T_2, 4 days	15	102	30		
" 7 "	23	97	36		
" 31 "	7	127	11		
1608, 7 "	14	43	56		
Acetone-dried pig heart (12 days)	81	126	78		

* 265 to 530 mγ without activity with tyrosine decarboxylase; the same quantity of pyridoxal phosphate gave full activity.

† Aspartic acid added to the cup, α-ketoglutarate tipped after 10 minutes. All the samples autolyzed 18 hours, 37°, and allowed to stand 5° in 0.1 M phosphate buffer, pH 7.5, for the days indicated.

Cotransaminase Activity of Pyridoxamine Phosphate

Dried Bacterial Cells—The glutamic-aspartic apotransaminase of *Streptococcus faecalis* R was prepared by two methods, first by the growth of vitamin B_6-deficient cells and second by resolution of the enzyme from cells grown in a complete medium, as described by Lichstein, Gunsalus, and Umbreit (2).

The apoenzyme from deficient cells was activated by pyridoxal or pyridoxamine phosphate to about an equal extent (Fig. 4), and to a lesser degree by pyridoxal or pyridoxamine in the presence of adenosine triphosphate (ATP). The cell-free enzyme prepared from dried cells and resolved was also activated by pyridoxal or pyridoxamine phosphate, though not by the unphosphorylated compounds even in the presence of ATP (Fig. 5). These data were interpreted as indicating the function of pyridoxamine phosphate as a coenzyme for transamination, in addition to pyridoxal phosphate as previously reported (1, 2), and thus furnished evidence in favor of Snell's hypothesis for the mechanism of coenzyme action (13).

TABLE II

Pyridoxal Phosphate and Pyridoxamine Phosphate with Purified Pig Heart Transaminase

Per Warburg cup, 0.5 ml. of 0.2 M phosphate, pH 7.3, 0.1 ml. of enzyme, coenzyme, and water to 1.7 ml.; 1st side arm, 0.25 ml. of 0.8 M aspartic acid,* 0.25 ml. of 0.4 M α-ketoglutarate; 2nd side arm, 0.5 ml. of aniline citrate.

Enzyme	Additions	Microliters oxalacetate formed per 10 min., 37°				
		None	Pyridoxal phosphate		Pyridoxamine phosphate†	
			per 10 γ		*per 10 γ*	
Sample 0, diluted 1:40‡	Incubate with asparate	4	91		12	
	" " α-ketoglutarate	4	56		9	
			per 1 γ	*per 5 γ*	*per 1 γ*	*per 5 γ*
	Tip both	4	13	36	9	11

In order to generalize upon the evidence for the intermediate function of pyridoxamine phosphate, five separate *Streptococcus faecalis* R cell preparations were tested (Table I). When tested as partially resolved cell-free preparations, four of these were activated, whereas the fifth, Sample R_6T_2, was not. Also, as shown in Table I, a sample of acetone-dried pig heart was partially resolved and could be activated by pyridoxal phosphate but not by pyridoxamine phosphate.

---

* Add one to the cup, tip the other from the side arm after 10 minutes.

† 500 mγ inactive in tyrosine decarboxylase, 50 mγ of pyridoxal phosphate give full activity.

‡ We wish to thank Doreen O'Kane for this enzyme sample; see (17).

The latter two observations cast serious doubt upon the direct mediation of pyridoxamine phosphate in transamination, since, if this substance were acting by direct mechanism, all cases should be positive.

Resolved Pig Heart Transaminase—To test critically the activity of pyridoxamine phosphate in transamination, it was felt that a purified enzyme, free of possible supplementary enzymes which catalyzed various side reactions, would be highly desirable. Therefore, the glutamic-aspartic transaminase of pig heart was resolved and purified (17). As shown in Table II, pyridoxal phosphate, but not pyridoxamine phosphate, was active with this enzyme. These data, in addition to the data with partially resolved acetone-dried pig heart enzyme, constitute evidence against pyridoxamine phosphate as a coenzyme of transamination. It should be recalled, however, that these data are subject to the limitation of chemical criteria for the identity of the pyridoxamine phosphate and must, therefore, remain tentative.[1]

DISCUSSION

The activity of pyridoxamine phosphate, prepared by heating pyridoxal phosphate with glutamic acid, for the glutamic-aspartic transaminase of dried bacterial cells and the lack of activity for the purified pig heart enzyme leave the evidence for the mechanism of coenzyme action in an anomalous state. While the extension of the data to several dried bacterial preparations strengthens the possibility, the single exception (Sample R_6T_2, Table I) constitutes stronger evidence against this mechanism.

Contradictory data from two sources constitute lack of substantiation of the pyridoxal phosphate-pyridoxamine phosphate mechanism and suggest that the Schlenk and Fisher (22) interpretation of the preliminary experiments of Umbreit, O'Kane, and Gunsalus (14) as conclusive evidence may be premature.

At present the following possibilities as to the disagreement in the data exist. (1) The proposed mechanism is incorrect. (2) The mechanism for the bacterial enzyme and pig heart enzyme is different; this seems highly unlikely. (3) The preparation obtained by heating glutamic acid with pyridoxal phosphate is not pyridoxamine phosphate, but is a compound which the bacterial cells can convert to an active compound, either to pyridoxamine phosphate or to pyridoxal phosphate.[1] Pyridoxamine phosphate, as such, is not active in the transaminase reaction, but may be converted into pyridoxal phosphate by supplementary enzymes present in the bacterial preparations. An analogy for this is found in the formation of coenzyme for tyrosine decarboxylase by resting suspensions of *Streptococcus faecalis* in the presence of pyridoxamine, provided that the cells are incubated with a keto acid before the decarboxylase activity is determined.

---

[1] Snell (personal communication) has now shown growth-promoting properties of pyridoxamine phosphate produced by heating pyridoxal phosphate with glutamic acid to coincide with those of the compound prepared in the Research Laboratories of Merck and Company by the direct phosphorylation of pyridoxamine.

The demonstration of Snell *et al.* (18, 19) by microbiological assay of a compound in natural material possessing the properties of pyridoxamine phosphate and its replacement by material prepared by heating pyridoxal phosphate with glutamic acid clarifies the natural occurrence of pyridoxamine phosphate and strengthens the evidence for the nature of the heated product used with the bacterial cell-free transaminase.

SUMMARY

Preparations of "pyridoxamine phosphate" obtained by heating pyridoxal phosphate with glutamic acid under the conditions suggested by Snell for the formation of pyridoxamine from pyridoxal yield preparations which are active in stimulating the glutamic-aspartic transaminase enzyme of dried cells of *Streptococcus faecalis* R.

These pyridoxamine phosphate preparations are not active with tyrosine decarboxylase, nor with purified glutamic-aspartic transaminase apoenzyme of pig heart, both of which are activated by pyridoxal phosphate.

The results of these findings are discussed and their implications pointed out.

BIBLIOGRAPHY

1. Gunsalus, I. C., and Bellamy, W. D., *J. Biol. Chem.*, **155**, 357 (1944).
2. Lichstein, H. C., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, **161**, 311 (1945).
3. Schlenk, F., and Snell, E. E., *J. Biol. Chem.*, **157**, 425 (1945).
4. Stokes, J. L., and Gunness, M., *Science*, **101**, 43 (1945).
5. Lyman, C. M., Moseley, O., Wood, S., Butler, B., and Hale, F., *J. Biol. Chem.*, **167**, 177 (1947).
6. Lyman, C. M., and Kuiken, K. A., *Federation Proc.*, **7**, 170 (1948).
7. Gunsalus, I. C., Umbreit, W. W., Bellamy, W. D., and Foust, C. E., *J. Biol. Chem.*, **161**, 743 (1945).
8. Umbreit, W. W., and Gunsalus, I. C., *J. Biol. Chem.*, **159**, 333 (1945).
9. Gunsalus, I. C., Bellamy, W. D., and Umbreit, W. W., *J. Biol. Chem.*, **155**, 685 (1944).
10. Baddiley, J., and Gale, E. F., *Nature*, **155**, 727 (1945).
11. Umbreit, W. W., Wood, W. A., and Gunsalus, I. C., *J. Biol. Chem.*, **165**, 731 (1946).
12. Wood, W. A., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, **170**, 313 (1947).
13. Snell, E. E., *J. Am. Chem. Soc.*, **67**, 194 (1945).
14. Umbreit, W. W., O'Kane, D. J., and Gunsalus, I. C., *J. Bact.*, **51**, 576 (1946).

15. Ames, S. R., Sarma, P. S., and Elvehjem, C. A., *J. Biol. Chem.*, **167**, 135 (1947).
16. Bellamy, W. D., Umbreit, W. W., and Gunsalus, I. C., *J. Biol. Chem.*, **160**, 461 (1945).
17. O'Kane, D. E., and Gunsalus, I. C., *J. Biol. Chem.*, **170**, 425 (1947).
18. Rabinowitz, J. C., and Snell, E. E., *J. Biol. Chem.*, **169**, 643 (1947).
19. McNutt, W. S., and Snell, E. E., *J. Biol. Chem.*, **173**, 801 (1948).
20. Bellamy, W. D., and Gunsalus, I. C., *J. Bact.*, **50**, 95 (1945).
21. Green, D. E., Leloir, L. F., and Nocito, V., *J. Biol. Chem.*, **161**, 559 (1945).
22. Schlenk, F., and Fisher, A., *Arch. Biochem.*, **12**, 69 (1947).

THE NATURE OF THE CIRCULATING THYROID HORMONE*

BY ALVIN TAUROG AND I. L. CHAIKOFF

(*From the Division of Physiology, University of California Medical School, Berkeley*)

(Received for publication, June 30, 1948)

Iodine exists in the thyroid gland in at least two well defined chemical entities, namely diiodotyrosine and thyroxine, which, according to Harington, account for practically all of the organic iodine in the gland ((2) p. 91). These two compounds are not present in a free form but are combined with other amino acids to form the characteristic thyroid protein, thyroglobulin.

Iodine is also a component of plasma, but its concentration there is so minute (about 5 γ per 100 cc.) that the chemical form in which it exists has eluded discovery. It was formerly believed that plasma iodine is also present as thyroglobulin (3, 4), but this view has been abandoned, mainly as a result of the studies of Trevorrow (5) and of Lerman (6). Despite the fact that crystalline thyroxine, when administered, produces in the mammalian organism all the known effects of thyroid tissue, investigators have nevertheless hesitated to assert that the circulating form of the thyroid hormone is thyroxine *per se*. This rejection is based on the following observations: (1) the failure of some investigators to account completely for the biological activity of thyroglobulin by its thyroxine content ((7, 8), ((9) p. 114), (2) the delayed response of animals to injected thyroxine ((2) p. 123, (10)), and (3) the failure of thyroxine to act *in vitro* (11, 12). These observations led Harington in 1935 (8, 13) to postulate that the circulating hormone is a peptide containing both thyroxine and diiodotyrosine. Recently, however, reevaluating this older evidence, Harington (14) has arrived at the conclusion that the peptide concept is an unnecessary complication and that thyroxine itself is probably the circulating hormone.

Two powerful tools, namely a refined method for determining small quantities of iodine (15) and the radioactive isotope of iodine (I^{131}), made possible a new attack on the problem of the nature of plasma iodine. The evidence provided by these means is presented here.

EXPERIMENTAL

The experiments presented here may be grouped conveniently as follows: (1) those dealing with the extractability of plasma iodine with butyl al-

---

* A preliminary report of some of the data presented here has already appeared (1). Aided by grants from the United States Public Health Service and the Committee on Endocrinology of the National Research Council.

cohol; (2) those dealing with the butyl alcohol extractability of thyroxine added to plasma; (3) the demonstration that the thyroid hormone of plasma labeled by means of radioactive iodine behaves exactly the same as added thyroxine carrier, as judged by the latter's recrystallization to constant specific activity and by its distribution between two immiscible solvents; (4) those dealing with the combination of thyroxine with plasma protein.

Extraction of Plasma Iodine with Butyl Alcohol

The organic solvent *n*-butyl alcohol has proved helpful in the determination of the thyroxine content of thyroid tissue. It was first used for this purpose in 1932 by Leland and Foster (16), who showed that it extracted all of the thyroxine from thyroid protein that had been subjected to strong hydrolysis with 2 N NaOH. Later several investigators (5, 17–19) used butyl alcohol for the fractionation of blood iodine, but their efforts led to no agreement as to the nature of the circulating thyroid hormone. This is not surprising in view of the difficulties encountered in measuring the small quantities of iodine involved in such experiments.

A sensitive and reliable method for the determination of plasma iodine, previously reported from this laboratory (15), enabled us to carry out butyl alcohol fractionation on smaller quantities of plasma than was previously possible and thus to achieve complete extraction with convenient volumes of solvent. The details of this procedure are described below.

3 cc. of heparinized plasma were added, with shaking, to 15 cc. of normal butyl alcohol (reagent grade) in a 50 cc. narrow necked centrifuge tube. The tube was stoppered (the rubber stopper had previously been treated with alkali and acid and then soaked in butyl alcohol) and shaken thoroughly. The butyl alcohol layer obtained by 10 minutes of centrifugation was quite clear and was transferred to a 125 cc. separatory funnel. The residue was reextracted twice, each time with 15 cc. of butyl alcohol, and the mixture centrifuged after each addition. The three clear butyl alcohol extracts were combined in the separatory funnel and shaken with 50 cc. of a reagent consisting of 4 N NaOH and 5 per cent Na_2CO_3. The latter reagent, introduced by Blau (20), extracts inorganic iodide and diiodotyrosine, but not thyroxine, from butyl alcohol. After the mixture was allowed to stand for a few hours, the lower aqueous layer was run out and the butyl alcohol fraction extracted a second time with 30 cc. of 4 N NaOH-5 per cent Na_2CO_3. This time the separation of the two layers was allowed to proceed for 15 hours, at the end of which time the butyl alcohol layer showed only a slight turbidity. The butyl alcohol was next transferred to the two-neck digestion flask used in the iodine determination and carefully concentrated to dryness under reduced pressure at 100°. The iodine deter-

mination was carried out on the residue as previously described by us for plasma iodine (15) except for the following modifications: (1) Hydrogen peroxide (1 cc. of a 1:20 dilution of Merck's superoxol) was added after the addition of the phosphorous acid (21). The slight color in the distillate previously encountered when the peroxide was added (15) did not appear in the present investigation. We attribute this to the more careful construction of the iodine still used in this later work. (2) It seemed desirable to add some non-iodine-containing organic material to the butyl alcohol residue in order to make it more comparable to the original plasma. For this purpose we used dried defatted muscle containing a minimum amount of iodine. Such muscle was obtained from rats that had been fed a low iodine diet containing 0.15 per cent propylthiouracil. In some of the samples wheat was added as organic carrier, since it was found in control runs that it gave exactly the same results as the dried muscle. Two samples of butyl alcohol were shaken with the alkali reagent and concentrated to dryness as described above; these served as reagent blanks.

The results obtained by this butyl alcohol fractionation are shown in Table I. Blood was obtained from normal human subjects; a sufficient quantity was removed from each to permit carrying out triplicate or quadruplicate analyses on each plasma sample. The total and protein-bound iodine content of plasma was also determined for each subject. The protein-bound fraction was determined on a zinc hydroxide precipitate which had been washed twice with redistilled water.

It is clear from Table I that 90 per cent or more of the iodine in plasma is extractable with butyl alcohol at room temperature. This may be taken to mean that the iodine in plasma is not stably bound to protein. These results should be contrasted with those in Table II, which shows the extent to which the iodine of fresh thyroid tissue can be extracted with butyl alcohol; only a small per cent was found soluble. Only after strong hydrolysis does thyroid iodine become markedly soluble in butyl alcohol. There can be little doubt therefore that the chemical form of the iodine in plasma is different from that in the thyroid gland and that some degradation of the thyroid protein molecule takes place before the hormone is secreted into plasma.

As is shown in Table I, some iodine can be reextracted from the butyl alcohol by shaking the latter with the 4 N NaOH-5 per cent Na_2CO_3 reagent. This iodine, amounting to about 10 to 15 per cent of that present in the butyl alcohol, should represent the combined inorganic iodide and diiodotyrosine contents of the extract. Thus the diiodotyrosine content of plasma is at most only about 10 per cent of total plasma iodine. In the gland, however, diiodotyrosine iodine represents at least 60 per cent of the total iodine (22). It is apparent therefore that there is a preferential release of thyroxine by the gland into the circulation; only a small fraction of the gland's iodine leaves the gland as diiodotyrosine.

TABLE I

Extractability of Plasma Iodine with Butyl Alcohol

Plasma source	Iodine in 3 cc. plasma					
	Total	Protein-bound	Butyl alcohol-extractable		Butyl alcohol-extractable, not reextractable with 4 N NaOH-5 per cent Na_2CO_3	
	γ	γ	γ	*per cent of total*	γ	*per cent of total*
Human female	0.188	0.183	0.174	93	0.150	80
“ male	0.165	0.165	0.162	98	0.135	82
“ “	0.168	0.174	0.147	88	0.156	93
“ “	0.195	0.198			0.158	81
“ “	0.162	0.156	0.153	94	0.126	78
“ “	0.153	0.150			0.123	82
“ “	0.168	0.156	0.162	96	0.123	73
Rat, pooled	0.094	0.096	0.093	99	0.081	84

TABLE II

Extractability of Rat Thyroid Iodine with Butyl Alcohol

Thyroids of ten large rats were pooled, minced with scissors, and duplicate portions taken for the following treatment.

Total I in gland	Trichloroacetic acid-soluble I*	Butyl alcohol-extractable I†	Butyl alcohol-extractable, not reextractable with 4 N NaOH-5 per cent Na_2CO_3
mg. per cent	*per cent of total*	*per cent of total*	*per cent of total*
17.3	6.9	3.4	2.4

* Thyroid tissue was homogenized with 1 cc. of 10 per cent trichloroacetic acid in a small glass tube.

† Thyroid tissue was homogenized with 1 cc. of butyl alcohol in a small glass tube. The residue was then extracted three times, each time with 3 cc. of butyl alcohol.

Behavior of Thyroxine Added to Plasma

The finding that approximately 80 per cent of the iodine in plasma can be extracted with butyl alcohol from which it cannot be reextracted with the 4 N NaOH-5 per cent Na_2CO_3 reagent suggested that this iodine is thyroxine. It therefore became of interest to compare the properties of this iodine fraction of plasma with those of crystalline thyroxine.

Butyl Alcohol Extraction—Crystalline thyroxine was added to human plasma in an amount comparable to that already present, and this treated plasma was subjected to the butyl alcohol extraction procedure described above. Approximately 80 per cent of the added thyroxine was recovered in the final butyl alcohol extract (Table III). This compares favorably with the solubility properties of the iodine originally present.

Protein Precipitation—From the results in Table I it is clear that practically all of the iodine in plasma precipitates with proteins when zinc hydroxide is used as the precipitating agent. When crystalline thyroxine is added to plasma, it too precipitates quantitatively with proteins, as shown in Table IV. These results are in accord with those reported previously by Trevorrow (5) and by Bruger and Member (23).

TABLE III

Butyl Alcohol Extractability of Crystalline Thyroxine Added to Human Plasma

Procedure	Iodine determined in 3 cc. plasma		Thyroxine I added per 3 cc. plasma*	Thyroxine I recovered	
	Initial value	After adding thyroxine			
	γ	γ	γ	γ	*per cent of that added*
Total iodine	0.168	0.336	0.168		
Extractable with butyl alcohol but not reextractable with 4 N NaOH-5% Na_2CO_3	0.123	0.252		0.129	77
Total iodine	0.174	0.393	0.219		
Extractable with butyl alcohol but not reextractable with 4 N NaOH-5% Na_2CO_3	0.129	0.300		0.171	78

* The difference between the third and second columns.

Dialysis—The iodine in plasma is not dialyzable, as is shown in Table V and as reported previously by Silver and Tyson (24). A small amount of thyroxine when added to plasma is not dialyzable, although in simple aqueous solution thyroxine will dialyze readily.

Extraction of Diiodotyrosine from Plasma to Which It Had Been Added—Crystalline diiodotyrosine was added to plasma and the mixture extracted with butyl alcohol as described above. The amount of iodine added as diiodotyrosine was approximately equal to the total iodine initially present in the plasma sample. Table VI shows that the addition of 5 to 6 γ per cent of diiodotyrosine iodine to plasma did not affect the value obtained for the iodine in the thyroxine fraction. The method described here, therefore, should prove valuable in distinguishing between thyroxine iodine and diiodotyrosine iodine in plasma.

Unfortunately, the presence in plasma of 17 γ per cent of the gallbladder dye, tetraiodophenolphthalein, did affect the thyroxine iodine value appreciably. The method described here is therefore not suitable for the determination of hormonal iodine in patients in which visualization tests of the gallbladder were recently carried out with this organic iodine compound.

It may be concluded from the experiments described in this section that crystalline thyroxine added to plasma behaves almost exactly like the naturally occurring iodine of plasma. Such experiments suggest very strongly that the iodine in plasma is mainly in the form of thyroxine loosely attached to plasma protein. More evidence on this point was obtained with the aid of radioactive iodine, to be described in the following section.

TABLE IV

Precipitation of Added Thyroxine Iodine with Plasma Proteins When Zinc Hydroxide Was Used As Precipitating Agent

Plasma source	Iodine initially present in 3 cc. plasma		Thyroxine I added	Iodine present in protein ppt.	Recovery of added thyroxine in protein ppt.
	Total	Protein-bound			
	γ	γ	γ	γ	*per cent*
Human	0.141	0.138	0.234	0.363	96
"	0.168	0.156	0.168	0.336	107
Dog	0.14		2.08	2.16	97

TABLE V

Dialysis of Thyroxine Added to Human Plasma

Iodine initially present in 3 cc. plasma		Thyroxine I added	Non-dialyzable I	Recovery of non-dialyzable thyroxine I	
Total	Protein-bound				
γ	γ	γ	γ	γ	*per cent of added thyroxine*
	0.147	0	0.156		
0.168	0.156	0	0.168		
0.141	0.138	0.234	0.367	0.229	98
0.168	0.156	0.168	0.327	0.171	102

Experiments with Radioactive Iodine

It has been shown in this laboratory that, after the injection of a carrier-free dose of radioiodine into rats that have been fed a low iodine diet, the isotope is rapidly taken up by the thyroid gland, converted there to organic iodine, and then released into the plasma (25). Within 24 hours, about 90 per cent of the radioactive iodine in plasma is protein-bound and nearly all of this percentage is presumably the thyroid hormone. This procedure thus permits one to obtain *labeled* thyroid hormone in its normal physiological state.

Butyl Alchol Extraction of Radioactive Iodine from Rat Plasma—Twelve large rats were injected with 80 microcuries of I^{131} and their blood removed 24 hours later and pooled. The plasma was separated, and 3 cc. portions were taken for determination of total iodine, protein-bound iodine, and butyl alcohol-extractable iodine (Table VII). Both chemical and radioactive measurements were made on all plasma samples. Since the chemical method involved the distillation of the iodine into an alkaline medium, a portion of this alkaline distillate was used for the radioactive measurement by simply evaporating a suitable aliquot in a porcelain milk-ashing dish. Radioactivity was determined by means of a thin mica window Geiger-Müller tube.

TABLE VI

Effect of Presence of Diiodotyrosine and Tetraiodophenolphthalein on Determination of Thyroxine Iodine Content of Human Plasma

Substance added	Iodine added per 3 cc. plasma	Iodine in butyl alcohol fraction prepared from 3 cc. of plasma	
		Original plasma	Plasma with added I
	γ	γ	γ
Diiodotyrosine	0.16	0.129	0.123
"	0.17	0.123	0.123
Tetraiodophenolphthalein*	0.51	0.123	0.345

* Trade name, Iodeikon.

TABLE VII

Extraction of Chemical and Radioactive Iodine from Plasma of Rats Injected 24 Hours Previously with Radioactive Iodine (I^{131})

	Iodine in 3 cc. plasma		Per cent of total I	
	Chemical I	Radioactive I	Chemical I	Radioactive I
	γ	*counts per sec.*		
Total I	0.096	348		
Protein-bound I	0.096	321	100	92
Butyl alcohol-extractable	0.093	250	97	72
" " but not reextractable with 4 N NaOH-5% Na_2CO_3	0.081	230	84	66

As is shown in Table VII, the butyl alcohol extractability of the iodine in rat plasma is quantitatively similar to that of human plasma. Almost all of the iodine passed into butyl alcohol, and about 84 per cent resisted further extraction with the 4 N NaOH-5 per cent Na_2CO_3 reagent. The results with the radioactive iodine were somewhat different. Only 72 per cent passed into the butyl alcohol, and 66 per cent remained in the butanol after the latter was subjected to extraction with alkali.

In seeking an explanation for the apparent discrepancy between the chemical and the radioactive data the following factors must be considered. (1) Not all of the iodine in the rat plasma had the same specific activity. If, for example, a small amount of diiodotyrosine-like iodine leaked into the plasma from the gland, this iodine would have a higher specific activity than the iodine of the thyroxine fraction (22). The injected iodide that was still present in the plasma would, of course, also have a much higher specific activity than the thyroxine iodine. (2) Inorganic iodide and diiodotyrosine, the compounds with the highest specific activity, are extractable from plasma with butyl alcohol to a lesser degree than thyroxine.

In the light of these considerations it would be expected that the chemical iodine in the plasma of the injected rats would be extracted with butyl alcohol to a greater extent than the radioactive iodine. Whether this alone accounts entirely for the discrepancy is difficult to say.

The possibility of some error (5 to 10 per cent) in the chemical method used here must also be considered in seeking to explain the discrepancy between the chemical and the radioactive data. Admittedly, the determination of 0.1 to 0.2 γ of iodine in the butyl alcohol extracts was not a simple matter, but it must be added that such analyses were always done in triplicate or quadruplicate. It seems unlikely to us that the average value of 80 per cent for the butyl alcohol-soluble, alkali-insoluble fraction of plasma iodine suffered from an error of more than 5 per cent.

Recrystallization of Thyroxine Carrier Added to Butanol Extracts—Butyl alcohol extracts, reextracted with the alkali reagent, were prepared from the plasma of rats injected 24 hours previously with 75 to 80 microcuries of I^{131}. 25 mg. of recrystallized thyroxine were added as carrier, and the butyl alcohol extract was concentrated to dryness on a boiling water bath under reduced pressure. The thyroxine in the residue was dissolved in hot 0.1 N K_2CO_3 and separated from a small amount of undissolved material by centrifugation. The thyroxine was then repeatedly recrystallized as follows: (1) The first crystallization was effected by the addition of glacial acetic acid to the hot K_2CO_3 solution of the residue. (2) The crystals obtained in this way were washed with water, dissolved in a minimum quantity of hot 0.1 N K_2CO_3, and then precipitated by cooling to 0°. (3) The crystals obtained in (2) were dissolved in an alkaline 70 per cent ethyl alcohol solution and precipitated by the addition of glacial acetic acid. (4) The fourth recrystallization was made from a dilute NaOH solution by the addition of glacial acetic acid.

The specific activity of the iodine of each batch of crystals (counts per second per microgram of I^{127}) was determined. The results are recorded in Table VIII. The constancy in the values indicates that the I^{131} in the butyl alcohol extract of rat plasma is in the same form as the material which underwent recrystallization, namely thyroxine. Such an experiment is not absolutely conclusive, however, since a compound very similar to thyroxine in its structure might conceivably continue to precipitate with thyroxine.

Solvent Distribution Experiment—To provide further evidence as to whether the radioactive iodine in the butyl alcohol extract of plasma was thyroxine, its distribution between two immiscible solvents was compared with that of added thyroxine carrier. The carrier was added to the butyl alcohol extract containing the radioactive iodine and the mixture concentrated to dryness on a boiling water bath under reduced pressure. The residue was dissolved in 15 cc. of 0.1 N NaOH and shaken with an equal volume of either butyl alcohol or isoamyl alcohol. The distribution of chemical and radioactive iodine between the organic and the aqueous phases is recorded in Table IX. It was found that the distribution of the I^{131} always paralleled that of the added thyroxine.

This parallelism is all the more striking, inasmuch as the distribution ratios varied from experiment to experiment. The variation suggests that a variable breakdown or transformation of the thyroxine occurs during the process of concentrating the alkaline butyl alcohol extracts to dryness. Despite these variations, however, the distribution ratios for radioactive and chemical iodine in any given experiment were about equal. Such results would be expected only when the radioactive iodine was of the same molecular species as the added carrier.

TABLE VIII

Specific Activity of Thyroxine Carrier after Successive Recrystallizations

See the text for details.

Experiment No.	Per cent of total I^{127} in BuOH fraction	Per cent of total I^{131} in BuOH fraction	Specific activity, counts per min. per γ I				
			Initial	After 1st recrystallization	After 2nd recrystallization	After 3rd recrystallization	After 4th recrystallization
1	84	66	2.64	2.58	2.58	2.47	2.53
2	84	66	2.57	2.25	2.20	2.28	2.28
3			2.90	2.69	2.81	2.74	2.70
4			2.89	2.69	2.71	2.66	2.63

Comparison of Behavior of Thyroxine and Thyroxine Peptide As Judged by Solvent Distribution Experiments—The evidence outlined above is best explained by the assumption that the iodine in plasma is mainly in the form of thyroxine loosely attached to protein. The question arises, however, whether a thyroxine-containing peptide is ruled out by the evidence at hand. To provide information on this point a thyroxine peptide was prepared by the procedure of Harington and Salter (26). Desiccated thyroid powder obtained from the Viobin Corporation was first hydrolyzed with pepsin (Merck) and then with pancreatin (Merck). A product was finally isolated which closely resembled that obtained by Harington and Salter. It contained 49.4 per cent I, 3.4 per cent N, 1.3 per cent amino N, and gave a strong nitrous acid color test for thyroxine. But it also gave a positive ninhydrin test, which indicates the presence of some free amino acid. If this free amino acid is assumed to have a molecular weight of 120 (average for ordinary amino acids), then it represents an impurity of about 6 per cent. If it is assumed to be free thyroxine, however, it would have amounted to about 40 per cent contamination because of the large molecular weight of thyroxine (777). The small amount of material available did not permit us to carry out all the analyses necessary to establish the true identity of this product. But it is safe to assume that it was composed mainly of thyroxine peptides, the ratio of total N to amino N (2.6) leading to the conclusion that it was a mixture of di- and tripeptides.

TABLE IX

Distribution of Crystalline Thyroxine Carrier and Radioactive Plasma Iodine between Two Immiscible Solvents

See the text for discussion.

Experiment No.	Amount of thyroxine carrier added	Concentration of I in organic solvent / Concentration of I in aqueous solution	
		Chemical I	Radioactive I
	mg.		
1*	10	1.67	1.78
2*	10	1.73	1.73
3*	1.5	1.43	1.35
4*	0.9	0.81	0.84
5†	10	0.069	0.072

* Solvent pair, 0.1 N NaOH-*n*-butyl alcohol.

† Solvent pair, 0.1 N NaOH-isoamyl alcohol.

A solvent distribution experiment was carried out with this preparation in the manner described above for thyroxine, except that the butyl alcohol extract, after the addition of the carrier, was not concentrated to dryness but shaken directly with 0.1 N NaOH. This avoided the possibility of chemical changes that might occur during the concentration. The results for both peptide carrier and thyroxine carrier are presented in Table X. It is evident that the radioactivity distributed itself very much like the thyroxine carrier but quite differently from the peptide carrier. *This demonstrates the sensitivity of this procedure in distinguishing between thyroxine and a closely related compound and lends further support to the view that the circulating thyroid hormone is actually thyroxine.*

It will be noted that the distribution ratios of thyroxine recorded in Table X are quite different from those in Table IX. In the former are recorded the results of the experiments in which the butyl alcohol extracts containing the thyroxine were not concentrated to dryness; the values found for the ratio of the concentration of I in butyl alcohol to the concentration of I in 0.1 N NaOH were much higher in these experiments than in those of Table IX. As already pointed out, the lower values observed in the experiments of Table IX may be attributed to some transformation of thyroxine which occurs while the butyl alcohol extract is being concentrated to dryness and which renders the iodine less soluble in butyl alcohol.

TABLE X

Comparison of Crystalline Thyroxine and Thyroxine Peptide Preparation in Solvent Distribution Experiment

Solvent pair, 0.1 N NaOH-*n*-butyl alcohol.

Sample No.	Carrier added	Amount of carrier added	$\frac{\text{Concentration of I in organic solvent}}{\text{Concentration of I in aqueous solution}}$	
			Chemical I	Radioactive I
		mg.		
1	Thyroxine	0.6	3.2	3.0
2	"	0.6	3.1	3.0
3	"	0.6	3.0	3.0
4	Peptide	1	1.5	3.1
5	"	1	1.5	3.2

Combination of Thyroxine with Plasma Proteins

The data so far presented lead to the conclusion that thyroxine in a loose combination with plasma protein is the circulating thyroid hormone. It seems of interest, therefore, to determine the particular fraction of plasma proteins with which thyroxine is combined.

Iodine Content of Plasma Protein Fractions—Salter and his coworkers (18, 27) have carried out iodine analyses on the various plasma protein fractions prepared by Cohn (28). The two albumin fractions, Nos. V and

VI, were found to contain 75 per cent of the protein-bound iodine. The concentration of iodine appeared to be greatest, however, in the α-globulin fraction.

These results have been confirmed by us (Table XI). The following plasma fractions were analyzed: crystalline bovine albumin, human albumin (Fraction V), human α-globulin (Fraction IV-1), human β-globulin (Fraction IV-3,4), and human γ-globulin (Fraction II). The α-globulin fraction had the highest concentration of iodine (1.9×10^{-4} per cent), the γ-globulin the lowest ($<1 \times 10^{-5}$ per cent). The iodine content of the albumin fraction was, unfortunately, too small for accurate analysis, and the value recorded is probably too low. Nevertheless the albumin fraction is the largest carrier of iodine because it constitutes the largest fraction of the plasma proteins. The values given in Table XI should, however, be regarded as preliminary observations; more complete results must await further refinements in the iodine method as well as a more abundant supply of pure α- and β-globulins.

TABLE XI

Iodine Content of Plasma Protein Fractions

See the text for discussion.

Fraction No.	Principal components	Iodine per 100 gm. protein	Approximate protein per 100 cc. plasma	Estimated I per 100 cc. plasma
		γ	gm.	γ
IV-1	α-Globulin β-Globulin	189	0.5	1
IV-3,4	β-Globulin α-Globulin	56	0.8	0.5
II	γ-Globulin	<10	0.8	0
V	Albumin	35	4	1.5
Crystalline bovine albumin	"	30		
I	Fibrinogen	<10	0.4	0

Thyroxine-Protein Combination—Many reports have dealt with the combination of various molecules with plasma proteins. Thus Roberts and Szego (29) reported that circulating estrogen is attached to plasma protein, probably the β-globulin fraction, whereas the combination of many organic anions with serum albumin has been studied by Klotz *et al.* (30), Teresi and Luck (31), and others. Davis (32) has discussed the physiological significance of the binding of molecules by plasma proteins.

In Table XII are presented our results on the binding of thyroxine by various plasma protein fractions. 100 mg. of each protein were dissolved or suspended in 10 cc. of a phosphate-saline (0.01 M phosphate-0.15 M

NaCl) buffer at pH 7.3. To 3 cc. of a protein solution in a small dialysis bag (Visking casing) was added 1 cc. of a standard thyroxine solution. Dialysis was carried out in a cold room (6–7°) against 100 cc. of phosphate buffer or distilled water. After 4 hours the external solution was replaced with a fresh 100 cc. portion and the dialysis continued for another 12 hours. The bag was rotated by motor during the dialysis. It is clear from the data in Table XII that albumin, α-globulin, and β-globulin are all capable of binding thyroxine to a high degree. γ-Globulin is much less potent as a thyroxine binder.

TABLE XII

Combination of Thyroxine with Plasma Proteins

Thyroxine was added to a 1 per cent protein solution in a phosphate-saline buffer at pH 7.3 and dialyzed. See the text for details.

Protein fraction	Thyroxine added	Dialyzed against	Per cent of added thyroxine remaining in dialysis bag
	mg.		
None	26.2	Distilled water	3.2
Albumin, Fraction V	21.4	" "	98
" " "	21.4	Phosphate-saline buffer, pH 7.3	82
α-Globulin, Fraction IV-1	21.4	Distilled water	86
" " "	21.4	Phosphate-saline buffer, pH 7.3	81
β-Globulin, " IV-3,4	25.2	Distilled water	91
" " "	24.5	Phosphate-saline buffer, pH 7.3	70
γ-Globulin, " II	25.2	Distilled water	58
" " "	24.5	Phosphate-saline buffer, pH 7.3	22

It may be concluded that plasma iodine is not confined to a single plasma protein fraction. Albumin, α-globulin, and, probably to a lesser extent, β-globulin share in binding the so called "protein-bound iodine" of plasma. γ-Globulin seems to play no rôle in this respect. It does seem, however, as pointed out by Salter (27), that the iodine in plasma is attached to the smaller protein molecules.

DISCUSSION

The concentration of "protein-bound" iodine in plasma is now widely used for the diagnosis of thyroid diseases (33–37).[1] This iodine is the fraction that is precipitated from plasma along with the proteins by such agents as zinc hydroxide, tungstic acid, or acetic acid plus heat, the inorganic iodine being freed from the protein precipitate by simple washing. Protein binding, however, is such a non-specific reaction that the finding of iodine in the plasma protein precipitate does not contribute much to an understanding of the chemical nature of this iodine.

---

[1] Chaney, A. L., private communication (1944).

Alcohol and acetone have been widely employed to fractionate the iodine of plasma ((9) p. 72, (38–40)). But such solvents do not achieve a clear cut separation of organic from inorganic iodine of plasma. Their inadequacy has been pointed out by Trevorrow (5), Salter ((9) p. 72), and others (23, 24, 38). Butyl alcohol is much more satisfactory for this purpose, since it has been successfully applied to the thyroid gland and since inorganic and non-thyroxine organic iodine can be reextracted from butyl alcohol by shaking this solvent with appropriate reagents.

The first to apply Leland and Foster's butyl alcohol fractionation procedure to blood iodine were Elmer *et al.* (17). They reported that after strong alkali hydrolysis (which destroyed part of the thyroxine present) 40 to 60 per cent of the organic iodine in the blood was thyroxine-like as judged by solubility properties. However, their values for normal human blood are high enough to cast suspicion upon their method for the determination of iodine.

Some time later, Trevorrow (5) also applied Leland and Foster's method to beef plasma and whole blood. She concluded that all of the iodine in plasma or whole blood could be directly extracted with butyl alcohol at room temperature and without previous hydrolysis. After reextraction of the butyl alcohol with 2 N NaOH, which served to remove inorganic and diiodotyrosine iodine, a good part of the iodine remained in the butyl alcohol. Her data did not permit quantitative conclusions regarding the percentage of total iodine in the thyroxine fraction.

Bassett, Coons, and Salter (18) also applied a butyl alcohol fractionation to plasma that had been subjected to pepsin hydrolysis. They reported that the thyroxine-like fraction in normal human plasma amounted to 73 per cent of the protein-bound iodine, but the range of values and number of patients studied were not given. Interestingly enough, approximately this same percentage was found in the thyroxine fraction in three cases of hyperthyroidism.

Somewhat different results were reported by Wilmanns in an extensive study of the iodine in normal, hyperthyroid, and hypothyroid blood (19). He treated whole blood with hot butyl alcohol and reported that only 65 per cent of the iodine could be removed from normal blood with this solvent, and of this only 28 per cent was not reextractable with 1 N NaOH. He concluded that there are at least two organic iodine fractions in whole blood: (1) free thyroxine (average 28 per cent) and (2) a stable protein-bound iodine fraction (average 34 per cent).

The results obtained in the present investigation confirm Trevorrow's observation that almost all of the iodine in plasma can be extracted with butyl alcohol at room temperature. This finding leads us to conclude that the iodine of plasma is not stably bound to protein and that its state thus differs from that in which iodine exists in the thyroid gland. The iodine of the gland becomes butyl alcohol-soluble only after strong hydrolysis. Not only is most of the iodine in plasma extractable with butyl alcohol, but, as Table I shows, only 10 to 15 per cent of it can be removed from this solvent with the reagent 4 N NaOH-5 per cent Na_2CO_3, which extracts inorganic iodine and diiodotyrosine, but not thyroxine, from butyl alcohol. In seven human subjects and in one sample of pooled rat plasma 73 to 93 per cent of the total plasma iodine (average 81 per cent) remained in the butyl alcohol extract after it had been treated with the alkali mixture. When crystalline thyroxine was added to plasma in physiological amounts and the mixture subjected to the butyl alcohol extraction procedure, 77 to 78 per cent of the thyroxine appeared in the final butyl alcohol extract. Such findings demonstrate that a large fraction (at least 80 per cent) of plasma iodine behaves like thyroxine in its solubility properties. The additional findings that thyroxine added to plasma precipitates quantitatively with the proteins (Table IV) and does not dialyze (Table V) also lend support to the view that the iodine in plasma is mainly in the form of thyroxine loosely attached to protein.

Further evidence that thyroxine exists in plasma is provided by the experiments with radioactive iodine (Tables VIII to X). Crystalline thyroxine carrier when added to the butyl alcohol extract of the plasma of rats injected with I^{131} showed a constant specific activity upon repeated recrystallization. Even more convincing, perhaps, was the finding that the radioactive iodine in the butyl alcohol extract distributes itself between two immiscible solvents in almost exactly the same manner as does added thyroxine carrier (Table IX), but quite differently from a thyroxine peptide carrier (Table X).

The results obtained here agree quite well with those of Bassett, Coons, and Salter (18), although these workers hydrolyzed the plasma protein with pepsin before extraction with butyl alcohol. Apparently, preliminary hydrolysis of the proteins does not appreciably affect the free thyroxine content of plasma.

Although Trevorrow reported that practically all of the iodine in small samples of blood or plasma could be extracted with butyl alcohol, she found that in the case of beef plasma a variable but large portion (42 to 73 per cent) of the butyl alcohol-soluble iodine could be reextracted with 2

N NaOH. Most of the values, however, were based on experiments in which large volumes of plasma were used and extraction with butyl alcohol was admittedly incomplete. Nevertheless, in view of this discrepancy between our results and Trevorrow's, the possibility of species variation must be considered.

In a recent paper dealing with the effects of thyroxine, thyroglobulin, and thyrotropin on tissue respiration (41), Williams and Whittenberger were led to the conclusion that the active form of the thyroid hormone is probably a thyroxine peptide. Their main evidence is that thyroxine had no calorigenic effect on liver tissue slices, whereas thyroglobulin[2] did. They also reported that the serum of myxedematous patients given thyroxine intravenously gradually acquired the capacity to raise the QO_2 of guinea pig liver slices incubated therein, the maximum effect being reached in 6 hours. The latter experiment was taken as evidence that injected thyroxine must undergo some process of activation before it can increase oxygen consumption. Peptide formation was suggested as a possible mode of activation. Salter also appears to favor the peptide hypothesis (27).

The results obtained by us favor thyroxine rather than thyroxine peptides as the chemical form of plasma iodine. If any activation of thyroxine is required before it can act on tissues, it seems unlikely that peptide formation is involved. More likely the solubility of thyroxine and its rate of penetration to the site of reaction are important.

Harington has reported immunological experiments which support the view that thyroxine itself circulates in the plasma (14). He found that antisera against artificial thyroxine-protein complexes protected rats against the characteristic response to a dose of thyroxine. He has also reviewed the evidence *against* the view that thyroxine itself is the normal circulating hormone and no longer finds it convincing. Nevertheless the whole question of the relation between the biological activity of thyroid preparations and their thyroxine content deserves accurate reinvestigation with proper attention to the effects of route of administration, solubility, relative activities of D- and L-thyroxine, accurate chemical thyroxine determinations, and method of biological assay.

The finding that the form in which thyroxine exists in plasma differs from that in the gland raises the question by what mechanism the hormone is released into the circulation. De Robertis and Nowinski (43) have reported the presence of a proteolytic enzyme in the gland which hydrolyzes thyroglobulin into smaller fragments. The concentration of this enzyme supposedly is increased in hyperthyroidism. Confirmation of these interesting findings would be welcomed as a further step toward an understanding of the workings of the thyroid gland.

---

[2] The claim that the addition of thyroglobulin increases the respiration of liver slices has not been confirmed (42).

We are indebted to the Cutter Laboratories for the supply of human plasma protein fractions, the Armour Laboratories for crystalline bovine albumin, E. R. Squibb and Sons for crystalline thyroxine, and the Viobin Corporation for the desiccated thyroid powder used in this investigation.
The technical assistance of Mr. David Feller is also gratefully acknowledged.

SUMMARY

The following evidence is presented in support of the view that the circulating thyroid hormone in the normal animal consists of thyroxine loosely attached to plasma protein.

1. The iodine of normal plasma is almost completely extractable with butyl alcohol at room temperature. Most of this iodine (73 to 93 per cent) remains in the butyl alcohol even after the latter is shaken with 4 N NaOH-5 per cent Na_2CO_3, a reagent which extracts diiodotyrosine and inorganic iodine, but not thyroxine, from butyl alcohol.

2. When crystalline thyroxine is added to plasma, it behaves like naturally occurring protein-bound iodine of plasma in the following respects: (*a*) Approximately 80 per cent of it remains in the butyl alcohol extract after treatment with the 4 N NaOH-5 per cent Na_2CO_3 reagent. (*b*) It precpitates quantitatively with plasma proteins when $Zn(OH)_2$ is used as the precipitating agent. (*c*) It does not dialyze.

3. Protein-bound iodine of rat plasma labeled with I^{131} follows thyroxine carrier quantitatively when the latter is repeatedly crystallized or when it is distributed between two immiscible solvents.

BIBLIOGRAPHY

1. Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, **171**, 439 (1947).
2. Harington, C. R., The thyroid gland, London (1933).
3. Hektoen, L., Carlson, A. J., and Schulhof, K., *J. Am. Med. Assn.*, **81**, 86 (1923).
4. Barnes, B. O., and Jones, M., *Am. J. Physiol.*, **105**, 556 (1933).
5. Trevorrow, V., *J. Biol. Chem.*, **127**, 737 (1939).
6. Lerman, J., *J. Clin. Invest.*, **19**, 555 (1940).
7. Means, J. H., Lerman, J., and Salter, W. T., *J. Clin. Invest.*, **12**, 685 (1933).
8. Harington, C. R., *Ergeb. Physiol. u. Exp. Pharmakol.*, **37**, 210 (1935).
9. Salter, W. T., The endocrine function of iodine, Cambridge (1940).
10. Thompson, W. O., Thompson, P. K., Brailey, A. G., and Cohen, A. C., *J. Clin. Invest.*, **7**, 437 (1929).
11. Gerard, R. W., and McIntyre, M., *Am. J. Physiol.*, **103**, 225 (1933).
12. Gordon, E. S., and Heming, A. E., *Endocrinology*, **34**, 353 (1944).
13. Harington, C. R., *Lancet*, **1**, 1261 (1935).

14. Harington, C. R., *Proc. Roy. Soc. London, Series B*, **132**, 223 (1944).
15. Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, **163**, 313 (1946).
16. Leland, J. P., and Foster, G. L., *J. Biol. Chem.*, **95**, 165 (1932).
17. Elmer, A. W., Luczynski, Z., and Scheps, M., *Compt. rend. Soc. biol.*, **115**, 1714 (1934).
18. Bassett, A. M., Coons, A. H., and Salter, W. T., *Am. J. Med. Sc.*, **202**, 516 (1941).
19. Wilmanns, H., *Z. ges.-exp. Med.*, **112**, 1 (1943).
20. Blau, N. F., *J. Biol. Chem.*, **102**, 269 (1933).
21. Chaney, A. L., *Ind. and Eng. Chem., Anal. Ed.*, **12**, 179 (1940).
22. Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, **169**, 49 (1947).
23. Bruger, M., and Member, S., *J. Biol. Chem.*, **148**, 77 (1943).
24. Silver, S., and Tyson, M. C., *J. Mt. Sinai Hosp.*, **12**, 701 (1945).
25. Chaikoff, I. L., Taurog, A., and Reinhardt, W. O., *Endocrinology*, **40**, 47 (1947).
26. Harington, C. R., and Salter, W. T., *Biochem. J.*, **24**, 456 (1930).
27. Salter, W. T., *Western J. Surg., Obs. and Gynec.*, **55**, 15 (1947).
28. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, **68**, 459 (1946).
29. Roberts, S., and Szego, C. M., *Endocrinology*, **39**, 183 (1946).
30. Klotz, I. M., Walker, F. M., and Pivan, R. B., *J. Am. Chem. Soc.*, **68**, 1486 (1946).
31. Teresi, J. D., and Luck, J. M., *J. Biol. Chem.*, **174**, 653 (1948).
32. Davis, B. D., *Am. Scientist*, **34**, 611 (1946).
33. Salter, W. T., Bassett, A. M., and Sappington, T. S., *Am. J. Med. Sc.*, **202**, 527 (1941).
34. Man, E. B., Smirnow, A. E., Gildea, E. F., and Peters, J. P., *J. Clin. Invest.*, **21**, 773 (1942).
35. Lowenstein, B. E., Bruger, M., and Hinton, J. W., *J. Clin. Endocrinol.*, **4**, 268 (1944).
36. Talbot, N. B., Butler, A. M., Saltzman, A. H., and Rodriguez, P. M., *J. Biol. Chem.*, **153**, 479 (1944).
37. Soley, M. H., and Miller, E. R., *Med. Clin. North America*, 1 (1948).
38. Boyd, E. M., and Clarke, E. L., *J. Biol. Chem.*, **142**, 619 (1942).
39. McClendon, J. F., and Foster, W. C., *J. Biol. Chem.*, **154**, 619 (1944).
40. Davison, R. A., Zollinger, R. W., and Curtis, G. M., *J. Lab. and Clin. Med.*, **27**, 643 (1942).
41. Williams, R. H., and Whittenberger, J. L., *Am. J. Med. Sc.*, **214**, 193 (1947).
42. Williams-Ashman, H. G., *J. Endocrinol.*, **5**, p. xc (1948).
43. De Robertis, E., and Nowinski, W. W., *J. Clin. Endocrinol.*, **6**, 235 (1946).

THE ISOLATION AND IDENTIFICATION OF 2,2′-DITHIOLISOBUTYRIC ACID FROM ASPARAGUS*

By EUGENE F. JANSEN

(*From the Enzyme Research Division, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture, Albany, California*)

(Received for publication, July 17, 1948)

A non-protein sulfhydryl substance occurs in edible asparagus. Fresh press-juice gives a test for both sulfhydryl and disulfide groups, so that an equilibrium apparently exists in the plant between the sulfhydryl and the disulfide (oxidized) form. However, when press-juice is exposed to atmospheric oxygen, the sulfhydryl form goes over completely to the oxidized form. Attempts to identify this substance as cystine or oxidized glutathione either by isolation or by polarographic analysis have been unsuccessful.[1]

It has now been found that the disulfide compound is acidic, thus permitting its isolation and purification by the extraction of concentrated acidified asparagus juice with butanol, removal of the substance from the butanol with sodium bicarbonate solution, subsequent acidification of the bicarbonate solution, and reextraction of the disulfide compound therefrom with butanol. A repetition of this distribution with several solvents gave a material which appeared to be pure. However, it was not found possible to crystallize the disulfide compound or its derivatives such as the methyl ester, since in the formation of the disulfide material from the sulfhydryl compound a relatively large polymer appeared to form. Even ethereal solutions of the disulfide compound became viscous on standing.

However, upon reduction with sodium in liquid ammonia, 2,2′-dithiolisobutyric acid (I) was isolated in pure crystalline form. The proof of the structure of this material was obtained from the accompanying series of reactions. Hydrogenolysis of 2,2′-dithiolisobutyric acid (I) with Raney's nickel (1) gave isobutyric acid (III) which was identified as the *p*-phenylphenacyl ester (2). Refluxing (I) with formaldehyde (3) gave a product whose analysis was in agreement with that of the previously undescribed 1,3-dithiane-5-carboxylic acid (II). The reaction of the sodium salt of the reduced product in liquid ammonia with methyliodide (4) gave dimethyl-2,2′-dithiolisobutyric acid (IV). This was shown by

---

* Enzyme Research Division Contribution No. 115. Report of a study made under the Research and Marketing Act of 1946.

[1] Potter, E. F., and Johnson, C. M., unpublished results.

the identity of the product so formed with synthetic dimethyl-2,2′-dithiolisobutyric acid, which was prepared from 2,2′-diiodoisobutyric acid (V). The 2,2′-diiodoisobutyric acid was synthesized by the oxidation of glycerol dichlorohydrin (VI) to dichloroacetone, cyanhydrin synthesis on this acetone derivative, subsequent hydrolysis to 1-hydroxy-2,2′-dichloroisobutyric acid, and finally reduction with fuming hydriodic acid (5).

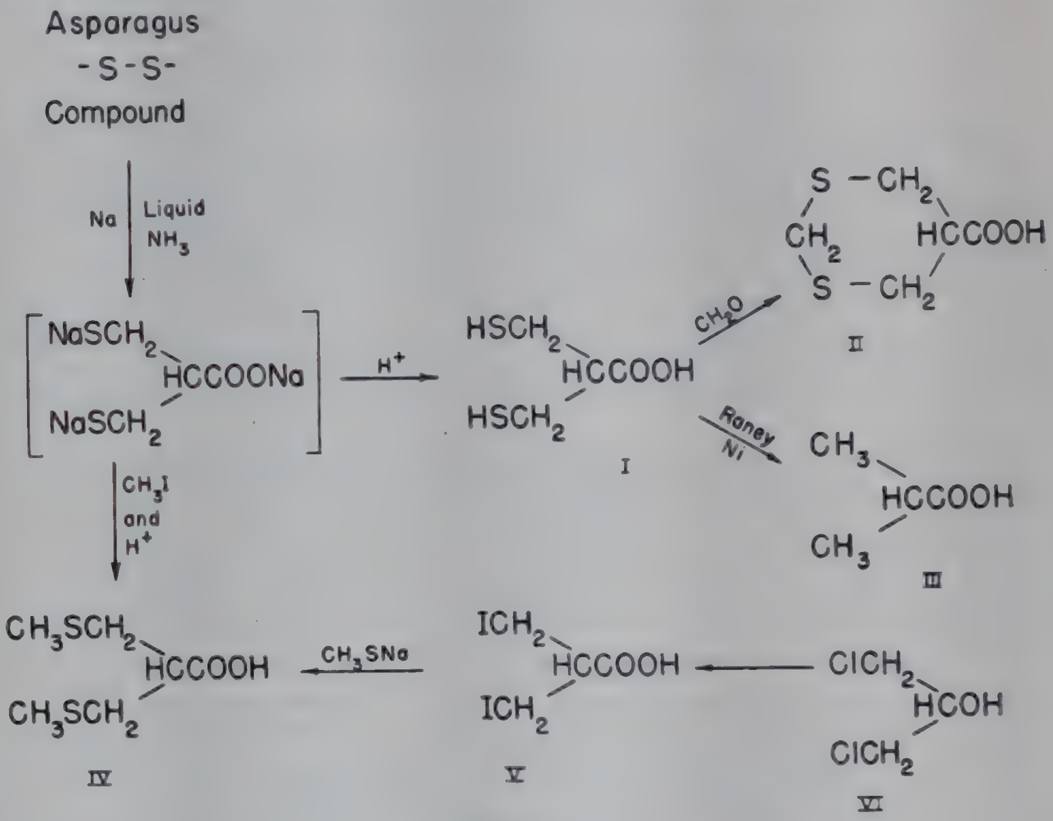

Asparagus
-S-S-
Compound
Na
Liquid NH₃
NaSCH₂
HCCOONa
NaSCH₂
H⁺
HSCH₂
HCCOOH
HSCH₂
I
CH₂O
S – CH₂
CH₂
HCCOOH
S – CH₂
II
Raney Ni
CH₃
HCCOOH
CH₃
III
CH₃I and H⁺
CH₃SCH₂
HCCOOH
CH₃SCH₂
IV
CH₃SNa
ICH₂
HCCOOH
ICH₂
V
ClCH₂
HCOH
ClCH₂
VI

FIG. 1

EXPERIMENTAL

Isolation of Asparagus Disulfide Compound—9 gallons of commercial asparagus concentrate,[2] weighing 40 kilos, were diluted with an equal volume of water. This solution, which was at pH 4.5, was extracted with 7 gallons of butanol by agitation for 20 minutes.[3] After standing for 2 hours the liquid phases were separated by means of a large Sharples supercentrifuge. The disulfide compound was then extracted from the butanol with 3 gallons of dilute sodium bicarbonate solution. After acidification of the bicarbonate extract, the disulfide compound was removed from the aqueous solution with a total of 4 gallons of butanol. The butanol solution was concentrated to a volume of 270 ml. *in vacuo* with a bath at 35° and a dry ice-alcohol trap. A small amount of insoluble material was filtered off and discarded. To the filtrate, 1300 ml. of benzene were added and the precipitate formed was filtered off. The benzene solution was then shaken thrice with 330 ml. of 5 per cent sodium bicarbonate solution; the bicarbonate solution was washed with benzene and then acidified to pH 2 with 10 per cent sulfuric acid. Some sticky black insoluble material was immediately removed by centrifugation and filtration. The disulfide compound was extracted from the water solution with 2 liters of ether in three batches. The pale yellow ether solution was dried over anhydrous sodium sulfate and concentrated to a volume of 100 ml. This ethereal solution, which contained 0.317 gm. per ml., was used in the subsequent work. Hence from 40 kilos of concentrate (which corresponds to approximately 1400 kilos of asparagus butts) 31.7 gm. of the disulfide compound were isolated. Since at pH 4.5 the disulfide compound was approximately one-third titrated,[4] this yield would have been greater if it were feasible to extract the concentrate with butanol at a lower pH.[3]

---

[2] The 66 brix concentrate (62.5 per cent solids) was prepared from asparagus butts by Libby, McNeill, and Libby of Sacramento, California.

[3] More of the disulfide compound could be extracted at a lower pH; however, relatively more impurities were likewise extracted.

Reduction of Disulfide Compound to 2,2′-Dithiolisobutyric Acid—To 175 ml. of liquid ammonia were added 13 ml. (4.1 gm.) of the ethereal solution of disulfide compound. Metallic sodium was added in small pieces until a relatively permanent blue color was obtained. As the reduction proceeded the material went into solution. The ammonia was allowed to evaporate spontaneously. The residue was taken up in 40 ml. of water and quickly acidified with concentrated hydrochloric acid to pH <2. The residue was allowed to stand overnight with 600 ml. of petroleum ether (b.p. 92–100°) and then reextracted with 200 ml. The petroleum ether was cooled to −18° for several days. Beautiful colorless crystals of 2,2′-dithiolisobutyric acid separated. Upon recrystallization from 800 ml. of petroleum ether 1.97 gm. of 2,2′-dithiolisobutyric acid were obtained corresponding to a 48 per cent yield on a solid basis. The product melted at 61–62°.

Analysis—$C_4H_8O_2S_2$
Calculated. C 31.56, H 5.30, S 42.12, mol. wt. 152
Found. " 32.3, " 5.30, " 41.6, neutral equivalent 152, mol. wt.[5] 155, equivalent weight by —SH titration[6] 77

---

[4] Unpublished results.

[5] The molecular weights were determined by the Clark modification (6) of the Signer isothermal distillation method.

[6] The —SH groups were titrated at pH 5.3 with *p*-chloromercuribenzoate with nitroprusside as an outside indicator according to the method of Hellerman *et al.* (7). The *p*-chloromercuribenzoate was standardized with a specially purified sample of cysteine hydrochloride. The value found agreed to within less than 2 per cent of the amount of *p*-chloromercuribenzoate weighed out.

Reaction of 2,2′-Dithiolisobutyric Acid with Formaldehyde—The conditions used for this reaction were essentially those which Armstrong and du Vigneaud used for the formation of djenkolic acid from cysteine (3). To a solution of 0.25 gm. (1.6 mM) of 2,2′-dithiolisobutyric acid in 50 ml. of 0.1 N HCl was added 0.14 ml. (1.9 mM) of formalin, and the reaction mixture was refluxed for 2 hours (the reaction proceeded very slowly at 70°). The reaction mixture was evaporated to dryness *in vacuo*, whereupon a crystalline residue was obtained. The crystals were recrystallized from 12 ml. of hot water, yielding 0.19 gm. of needles melting at 143–146°. This yield corresponds to 70 per cent of the theoretical for the conversion to 1,3-dithiane-5-carboxylic acid. Upon recrystallization from 10 ml. of hot water the product melted at 146–148°. The analyses were in agreement with the composition of 1,3-dithiane-5-carboxylic acid.

Analysis—$C_5H_8O_2S_2$

Calculated.	C 36.56, H 4.91, S 39.04, mol. wt. 164
Found.	" 36.7, " 5.04, " 39.8, neutral equivalent 161

This material no longer gave a nitroprusside test upon treatment with potassium cyanide. On an equivalence basis, it gave 65 per cent of the color that methionine gives in the Axelrod modification (8) of the McCarthy-Sullivan method for methionine (9). Axelrod[7] has shown that all the substances tested which contain a sulfur-methylene group gave this reaction.

Hydrogenolysis of 2,2′-Dithiolisobutyric Acid to Isobutyric Acid—The procedure used for the hydrogenolysis of 2,2′-dithiolisobutyric acid was the same that Mozingo *et al.* (1) employed for the hydrogenolysis of several sulfur compounds. To a suspension of 10 gm. of Raney's nickel (10) in 125 ml. of ethanol, 0.5 gm. (3.3 mM) of 2,2′-dithiolisobutyric acid and 75 ml. of water were added and the reaction mixture was refluxed for 2 hours. The nickel was filtered off and washed with 500 ml. of 0.2 N and 100 ml. of 1 N NaOH. The filtrate and washings were combined and the alcohol was distilled off of the alkaline solution. The aqueous solution was acidified with sulfuric acid and the isobutyric acid was steam-distilled. After neutralization of the distillate it was concentrated *in vacuo* to approximately 5 ml. and then made slightly acidic with 1 N hydrochloric acid. To this solution were added 0.907 gm. (3.3 mM) of *p*-phenylphenacyl bromide and 10 ml. of ethanol, and the reaction mixture was then refluxed for 1 hour (2), whereupon an oil separated. Sufficient 60 per cent ethanol was added to dissolve the oil when hot. On cooling overnight at 5°, 0.60 gm. of crystalline *p*-phenylphenacyl ester of isobutyric acid was obtained (65 per cent of theoretical), melting at 70–80°. After two recrystallizations from 60 per cent ethanol the melting point was constant at 88–90°.

---

[7] Axelrod, B., unpublished results.

The melting point of a mixture with an authentic sample of the ester was the same.

Analysis—$C_{18}H_{18}O_3$. Calculated. C 76.57, H 6.43
Found. " 76.3, " 6.45

Preparation of p-Phenylphenacyl Ester of Isobutyric Acid—Since this ester has not previously been described, it was necessary to prepare it for identification purposes. For this purpose the method of Drake and Bronitsky (2) was employed with 0.01 mole of isobutyric acid and an equivalent amount of *p*-phenylphenacyl bromide. The procedure was the same as above. After two crystallizations, the melting point was constant at 88–90°.

Analysis—$C_{18}H_{18}O_3$. Calculated. C 76.57, H 6.43
Found. " 76.4, " 6.26

Formation of Dimethyl-2,2′-Dithiolisobutyric Acid from Disulfide Compound—A metallic sodium reduction of 15 ml. of the ethereal solution of the disulfide compound was carried out as above. After the reduction was complete, 15 ml. of methyl iodide were slowly added to the liquid ammonia solution (4). The ammonia was allowed to evaporate spontaneously. The residue was dissolved in water and acidified with dilute hydrochloric acid to pH 2.0 and then evaporated *in vacuo* to dryness. The residue was extracted with 400 ml. of benzene, which dissolved the dimethyl-2,2′-dithiolisobutyric acid completely. Upon removing the benzene, 3.14 gm. of an oily product were obtained, corresponding to a yield of 60 per cent of theoretical. The oil was dissolved in 200 ml. of petroleum ether (b.p. 92–100°) at 25° and cooled to $-18°$, whereupon the dimethyl-2,2′-dithiolisobutyric acid crystallized out in elongated rectangles. After two more crystallizations the melting point was constant at 23.5–25°. The melting point of a mixture with synthetic dimethyl-2,2′-dithioliso-butyric acid was identical.

Analysis—$C_6H_{12}O_2S_2$
Calculated. C 39.97, H 6.71, S 35.57, CH_3 16.67, mol. wt. 180
Found. " 40.0, " 6.83, " 34.9[8] CH_3[9] 14.3, neutral equivalent 179, mol. wt.[5] 183

---

[8] These sulfur determinations were made by the Potter and Jones modification (11) of the Pollock and Partansky permanganate method (12), since this method is better suited for the determination of sulfur in substances which are oils at room temperature than is the Parr bomb peroxide method.

[9] The methyl determinations were carried out according to the Baernstein method for methionine (13) which gives results 6 per cent low (14). Substances other than

The dimethyl-2,2′-dithiolisobutyric acid gave 1.44 times as much color as did an equivalent amount of methionine in the modified (8) McCarthy-Sullivan method.

Synthesis of Dimethyl-2,2′-Dithiolisobutyric Acid—2,2′-Diiodoisobutyric acid was prepared from glycerol dichlorohydrin according to the directions of Glattfeld and Schneider (5), except that the cyanhydrin synthesis was carried out with the sodium bisulfide addition product of dichloroacetone and potassium cyanide (15) instead of dichloroacetone and liquid hydrogen cyanide.

To 125 ml. of a cold solution of sodium ethylate, prepared by reaction of 0.5 gm. (0.022 mole) of metallic sodium with ethanol, were added 9.5 gm. (0.195 mole) of methyl mercaptan. After a few minutes 2 gm. (0.006 mole) of 2,2′-diiodoisobutyric acid were added and the reaction mixture was allowed to warm to room temperature for 1 hour and then was refluxed for 6 hours. After acidification the reaction mixture was evaporated to dryness *in vacuo*. The residue was extracted with 100 ml. of warm petroleum ether (b.p. 92–100°) and the extract filtered. On cooling to −18° overnight only an oil separated. This oil was removed and the mother liquor was evaporated to a volume of 25 ml. On cooling again to −18°, 0.311 gm. of crystals melting at 23–24.5° was obtained. The yield of crystalline dimethyl-2,2′-dithiolisobutyric acid thus was 30 per cent of theoretical. After two recrystallizations, which were necessary for removal of the yellow color, the melting point was 23.5–25°.

Analysis—$C_6H_{12}O_2S_2$
Calculated. C 39.97, H 6.71, S 35.57, CH_3 16.67, mol. wt. 180
Found. " 39.8, " 6.76, " 35.0,[8] CH_3[9] 14.3, neutral equivalent 179

The synthetic dimethyl-2,2′-dithiolisobutyric acid under investigation gave the same amount of color in the modified McCarthy-Sullivan methionine method as did the substance prepared from the natural product.

DISCUSSION

2,2′-Dithiolisobutyric acid appears to be the first dithiol substance reported from a natural source. Because of its apparent similarity to British anti-lewisite (2,3-dimercaptopropanol) its rôle in the metabolism of asparagus may well be as an enzyme regulator in that it might control heavy metal inhibition. Or, since freshly pressed asparagus juice gives a test for both free sulfhydryl and disulfide groups, it may function as a

---

methionine may well give still lower results, particularly those containing two—SCH_3 groups. Hence these results cannot be considered as quantitative but only as establishing that two —SCH_3 groups occur in this compound.

hydrogen transfer substance similar to glutathione. The effect of this substance on respiration and on isolated enzymes will be investigated.

Other sulfur compounds which might be called unusual have been isolated from plant sources. Haagen-Smit *et al.* (16) have isolated methyl-β-methyl thiolpropionate from the volatile constituents of pineapple. Kröner and Wegner (17) have obtained concentrates of sulfur compounds from potatoes which are responsible for at least part of the odor and taste. It is noteworthy that one of the fractions of such compounds gave a direct reaction with silver foil. Hence there exists a variety of sulfur-containing substances in plants which undoubtedly contribute to the taste. Alterations in these substances during processing or storage of fruits and vegetables would most likely lead to off flavor formation.

Dithiolisobutyric acid, which for a mercaptan has relatively slight odor, apparently is not the precursor of the characteristic odor of the urine resulting after the ingestion of asparagus, since no odor resulted when two individuals took 10 mg. each of dithiolisobutyric acid orally.

SUMMARY

2,2′-Dithiolisobutyric acid has been isolated from asparagus by reduction of the oxidized form with metallic sodium. Proof of the structure of this substance was obtained from the following reactions. Hydrogenolysis of the 2,2′-dithiolisobutyric acid with Raney's nickel gave isobutyric acid (identified as the *p*-phenylphenacyl ester). Reaction with formaldehyde led to the formation of 1,3-dithiane-5-carboxylic acid. The sodium salt of the reduced disulfide substance obtained from asparagus, when treated in liquid ammonia with methyl iodide, gave dimethyl-2,2′-dithiolisobutyric acid. The acid so obtained was identical with dimethyl-2,2′-dithiolisobutyric acid prepared synthetically from glycerol dichlorohydrin.

I am indebted to Dr. A. K. Balls for many helpful suggestions during the course of this work, and to Dr. O. H. Emerson, Dr. B. Axelrod, and Miss R. Jang of the Enzyme Research Division, and Mr. Arthur Bevenue and Mr. L. M. White of the Western Regional Research Laboratory for many of the analyses reported.

BIBLIOGRAPHY

1. Mozingo, R., Wolf, D. E., Harris, S. A., and Folkers, K., *J. Am. Chem. Soc.*, **65**, 1013 (1943). Fonken, G. S., and Mozingo, R., *J. Am. Chem. Soc.*, **69**, 1212 (1947).
2. Drake, N. L., and Bronitsky, J., *J. Am. Chem. Soc.*, **52**, 3715 (1930).
3. Armstrong, M. D., and du Vigneaud, V., *J. Biol. Chem.*, **168**, 373 (1947).
4. du Vigneaud, V., Dyer, H. M., and Harmon, J., *J. Biol. Chem.*, **101**, 719 (1933).

5. Glattfeld, J. W. E., and Schneider, J. M., *J. Am. Chem. Soc.*, **60,** 415 (1938).
6. Clark, E. P., *Ind. and Eng. Chem., Anal. Ed.*, **13,** 820 (1941).
7. Hellerman, L., Chinard, F. P., and Deitz, V. R., *J. Biol. Chem.*, **147,** 443 (1943).
8. Balls, A. K., Walden, M. K., and Thompson, R. R., *J. Biol. Chem.*, **173,** 9 (1948).
9. McCarthy, T. E., and Sullivan, M. X., *J. Biol. Chem.*, **141,** 871 (1941).
10. Mozingo, R., in Organic syntheses, New York, **21,** 15 (1941).
11. Potter, E. F., and Jones, C. B., *Ind. and Eng. Chem., Anal. Ed.*, **15,** 15 (1943).
12. Pollock, R. N., and Partansky, A. M., *Ind. and Eng. Chem., Anal. Ed.*, **6,** 330 (1934).
13. Baernstein, H. D., *J. Biol. Chem.*, **115,** 25 (1936).
14. Kassell, B., and Brand, E., *J. Biol. Chem.*, **125,** 145 (1938).
15. Wagner, E. C., and Baizer, M., in Organic syntheses, New York, **20,** 43 (1940).
16. Haagen-Smit, A. J., Kirchner, J. G., Deasy, C. L., and Prater, A. N., *J. Am. Chem. Soc.*, **67,** 1651 (1945).
17. Kröner, W., and Wegner, H., *Naturwissenschaften*, **30,** 586 (1942).

DIPHOSPHOPYRIDINE NUCLEOTIDE PYROPHOSPHATASE

By ARTHUR KORNBERG* and OLOV LINDBERG†

(*From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis*)

(Received for publication, May 18, 1948)

Enzymatic splitting of diphosphopyridine nucleotide (DPN)[1] by microorganisms, plants, and animal tissues has been known for many years, but the precise nature of this reaction is not well understood (1). Ohlmeyer (2) first observed that adenylic acid (5-phosphoadenosine) appeared when DPN was incubated with yeast maceration juice. Later Heiwinkel (3) identified adenylic acid as a product of the action of sweet almond press juice on DPN. Although free nicotinamide did not appear, the presumed nicotinamide mononucleotide could not be isolated. Das and von Euler (4, 5) found inorganic orthophosphate as a product of DPN destruction in animal tissues, but there was no indication as to the mechanism of this reaction. Handler and Klein (6) recovered free nicotinamide quantitatively as a product of DPN destruction by brain, liver, kidney, and muscle preparations from rabbits, rats, and dogs, and concluded that the nicotinamide-ribose linkage was the primary and principal site of cleavage in these tissues.

In the course of studies of oxidative phosphorylation, we observed that washed particles of rabbit kidney converted added DPN to adenosine triphosphate (ATP). In the absence of an oxidizable substrate to provide an energy source for phosphorylation, adenylic acid was formed in place of ATP. The other product of DPN splitting was tentatively identified, after partial purification, as nicotinamide ribose phosphate. These and related observations make it probable that in such rabbit kidney preparations cleavage of the pyrophosphate bond is the predominant mechanism of DPN splitting. Almost all of the DPN-splitting activity of rabbit brain is due to nucleosidase activity.

Methods

Washed Particles of Kidney Cortex—Kidneys of a young rabbit were chilled on ice. All subsequent operations were performed at 2°. The cortex was homogenized in 15 ml. of 0.1 M potassium phosphate buffer of pH 7.7 and centrifuged at 1500 R.P.M. for 5 minutes to remove coarse particles. The supernatant fluid was centrifuged at 10,000 R.P.M. for 30 minutes, and the particles obtained were washed three times with 1 per cent KCl and centrifuged each time at 10,000 R.P.M. for 15 minutes. They were suspended in 5 ml. of 0.1 M potassium phosphate buffer of pH 7.7, or in 5 ml. of 0.1 M $NaHCO_3$ (saturated at 22° with 5 per cent CO_2 + 95 per cent N_2). 1 ml. of $MgCl_2$ (0.03 M), 1 ml. of NaF (0.1 M), and water to 10 ml. were added. This kidney particle suspension contained 30 to 40 mg. of protein per ml. (Washed particles from rabbit brain were similarly prepared.) Unwashed homogenates in 0.1 M potassium phosphate buffer of pH 7.7 were used in some experiments.

---

* Present address, National Institute of Health, Bethesda 14, Maryland.

† Supported by fellowships of the Lilijewalchs Foundation, Hierta Retzius Foundation, and the Swedish government. Present address, Wenner Grens Institut, Stockholm, Sweden.

[1] Nicotinamide-ribose-phosphate-phosphate-ribose-adenine.

Diphosphopyridine Nucleotide—DPN of 0.60 to 0.72 purity was prepared according to Williamson and Green (7) with minor modifications. $DPNH_2$ of purity 0.50 was prepared by Ohlmeyer's method (8).

Determinations—Incubations were carried out at 37°, with shaking, in air or O_2. Usually the reaction was stopped by trichloroacetic acid; for $DPNH_2$ determinations, however, the reaction was stopped by heating for 3 minutes at 100°. DPN was determined by the method of Cori, Slein, and Cori (9) at 340 mμ in the Beckman spectrophotometer. As an occasional check that the optical density increments were due only to $DPNH_2$, pyruvate and lactic dehydrogenase[2] were added when the reaction was complete. The densities invariably returned to the initial values. $DPNH_2$ was determined in the lactic dehydrogenase system as described by Kubowitz and Ott (10).

Compounds containing the nicotinamide-ribose (N-R) moiety were determined by the fluorometric method of Huff and Perlzweig (11) in the Coleman photofluorometer, with DPN as a standard. Under the conditions of the method, the formation of a highly fluorescent condensation product with acetone is specific for pentavalent N^1-substituted nicotinamide derivatives. In expressing results, it has been assumed arbitrarily that N-R-containing compounds arising from DPN splitting have a molar fluorescence equal to that of DPN.[3]

Phosphate was determined by the method of Fiske and Subbarow (12). Acid-labile phosphate was taken to be the phosphate liberated after 20 minutes hydrolysis in 1 N H_2SO_4 at 100°. Acid-stable phosphate was determined as the additional phosphate liberated after ashing with an H_2SO_4-HNO_3 mixture. The procedure of Cohn and Kolthoff (13) was adapted for the estimation of inorganic pyrophosphate. Pentose was determined by the method of Mejbaum (14) and adenylic acid by the method of Kalckar (15).

---

[2] Purified from rabbit muscle extract.

[3] DPN gave 1.4 to 1.9 times as much fluorescence, expressed on a molar basis, as N^1-methylnicotinamide chloride. The latter compound was kindly supplied by Dr. V. A. Najjar.

Results

Splitting of DPN by Rabbit Kidney and Recovery of Adenylic Acid—Preparations of washed particles of rabbit kidney invariably split added DPN. This destruction was generally associated with little or no decrease in concentration of the N-R moiety. Of twenty preparations incubated for a time interval sufficient to destroy 50 per cent or more of the DPN, the N-R value was decreased by more than 20 per cent in only four. The N-R value was increased by more than 10 per cent in two, and remained nearly constant (±10 per cent) in ten. These findings will be considered again later in this report.

The loss of DPN was accompanied by the appearance of nearly equivalent amounts of adenylic acid. Thus, after 15 and 30 minutes incubation, 1.75 and 2.44 micromoles of DPN were lost and 1.36 and 2.36 micromoles of adenylic acid, respectively, were recovered. Recoveries of adenylic acid after longer incubation periods were less complete, owing to the splitting of adenylic acid by nucleotidase.

The DPN-splitting activity of kidney particles was roughly proportional to their concentration. The activity was destroyed by heating at 100° for 2 minutes and was not significantly altered by omission of $MgCl_2$, NaF, or phosphate.

Phosphate Balance and Separation of Reaction Products of DPN Splitting in Non-Respiring System—Without added substrate, there was practically no oxygen uptake by kidney particles, and the values for inorganic ortho-, acid-labile, and total organic phosphate were essentially unchanged during the splitting of DPN.[4]

Fractionation of the reaction products with mercury and lead resulted in partial separation of the three major components, adenylic acid, nicotinamide mononucleotide, and unsplit DPN (Table I). The incubation mixture was treated essentially as described below under "Purification of nicotinamide mononucleotide" through the mercury precipitation step. The insoluble mercury salts were freed of mercury and then fractionated with lead at pH 6.4. Adenylic acid was recovered as the predominant component of the insoluble lead salts, while DPN (unsplit) was recovered as a soluble lead salt. The soluble mercury fraction contained only small amounts of adenylic acid and DPN and consisted mainly of an N-R-containing compound. After refractionation with mercury, the latter fraction was found to contain phosphate, pentose, and N-R in roughly equivalent concentrations.

---

[4] While these results indicate that no significant amount of phosphate was liberated or esterified, small changes in phosphate concentration may have been obscured, since these experiments were carried out in 0.05 M phosphate buffer. In experiments with CO_2-bicarbonate buffer, up to 28 per cent of the phosphate content of the split DPN was liberated as inorganic orthophosphate. This indicates that some nucleotidase was present in these kidney preparations.

Stimulation of Respiration of Kidney Particles by DPN—The oxygen consumption and esterification of inorganic orthophosphate by rabbit kidney particles were markedly stimulated by DPN (Fig. 1, Table II). The rate of oxygen consumption in the presence of DPN showed no significant lag phase and remained constant throughout the period of observation. The stimulation of oxygen uptake by adenylic acid was somewhat less than with DPN and was not maintained so well. That the lesser effectiveness of adenylic acid was not due to a concomitant inhibitory effect is indicated by the rate of oxygen consumption with DPN and adenylic acid together, essentially the same as that for DPN alone and well maintained.

Table I

Separation of Reaction Products of DPN Splitting in Non-Respiring System

The incubation mixture contained 20.0 ml. of kidney particles suspended in 0.05 M phosphate buffer and 5.0 ml. of neutralized DPN solution. The values are expressed in micromoles.

	Adenylic acid	N-R[*]	DPN
Incubation mixture at 0 min.[†]	0	211	211
" " " 120 min.[†]	142	211	84
Mercury salt, soluble	9	95	6
" " insoluble; lead salt, insoluble	109	9	3
" " " " " soluble	4	79	70

[*] Nicotinamide-ribose moiety.

[†] Trichloroacetic acid filtrate.

With graded amounts of DPN (Table II), the oxygen consumption was increased to a maximum value. A limit of phosphate esterification was not reached in these experiments and all the phosphate taken up was easily hydrolyzable in acid. Fractionation of the reaction products with barium will be considered below.

Phosphate Balance and Separation of Reaction Products of DPN Splitting in Respiring System—The addition of an oxidizable substrate, such as glutamate, to kidney particles resulted in active oxygen consumption and phosphate esterification. In the experiment summarized in Table III, approximately half the orthophosphate disappeared and could be accounted for as an acid-labile ester. Fractionation of the esterified phosphate indicated the presence of two or possibly three components. The neutralized trichloroacetic acid filtrate of the reaction mixture was treated with an excess of barium acetate and the pH was adjusted to 6.9. The precipitate was dissolved in acid, the barium removed with H_2SO_4, and the pH buffered at 3.6. Addition of cadmium acetate, according to Cohn and Kolthoff (13), yielded a crystalline precipitate with only slight absorption at 260 mμ and can be presumed to be inorganic pyrophosphate (16). ATP is almost completely soluble as a cadmium salt at pH 3.6. Analysis of the phosphate ester not precipitated by cadmium indicated a composition consistent with that of ATP. Analysis of the barium precipitate obtained by raising the pH to 8.4 also indicated ATP as the predominant organic phosphate ester. The presence of ATP in both fractions was checked qualitatively by the phosphorylation of glucose in the hexokinase reaction. The barium salts soluble at pH 8.4 contained almost all of the N-R and a small amount of acid-labile phosphate ester. The over-all recoveries of phosphate, pentose, and N-R were in fair agreement with the starting concentrations in the form of DPN.

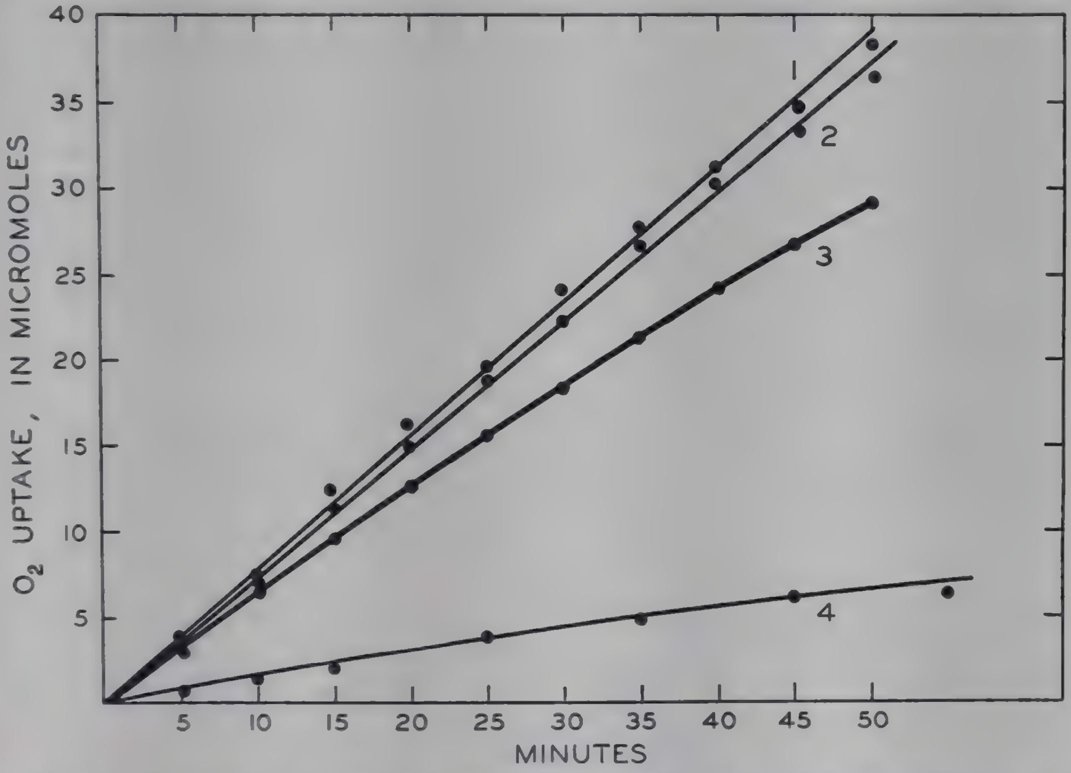


Fig. 1. Stimulation of respiration of kidney particles by DPN and adenylic acid. Curve 1, 2.5 micromoles of DPN; Curve 2, 2.5 micromoles of DPN + 2.5 micromoles of adenylic acid; Curve 3, 2.5 micromoles of adenylic acid; Curve 4, no additions. Each vessel contained 1.0 ml. of kidney particles, 130 micromoles of glutamate, the additions noted above, and water to make a final volume of 1.75 ml. Incubated in O_2 at 37°.

The results of Table II are consistent with those of Table III, except that the recovery of ATP was incomplete because the barium precipitate formed between pH 7 and 8.5 was not analyzed. Of some interest is the relative constancy of the acid-labile phosphate fraction precipitated by barium at pH 7, in which inorganic pyrophosphate is a prominent component (Table II). Possibly significant with respect to the question of whether nicotinamide mononucleotide is phosphorylated are the amounts of acid-labile phosphate in the barium salts soluble at pH 8.5, in which the N-R-containing compound is found (Tables II and III).

TABLE II

Effect of DPN on Oxygen Consumption and Phosphate Esterification

Each vessel contained 1.0 ml. of kidney particles in phosphate buffer, 130 micromoles of glutamate, and 4.2 micromoles of DPN at pH 7.7 in a volume of 1.3 ml. Incubation 60 minutes in O_2 at 37°. Corrections were made for the time elapsed in equilibration and fixation. Each sample was fixed with trichloroacetic acid and the filtrate fractionated with barium. Analyses were performed on the barium salts insoluble at pH 7 and on the barium salts soluble at pH 8.5. A barium precipitate which formed between pH 7 and 8.5 was not analyzed. The values are expressed in micromoles.

DPN added	4.2*	0	0.8	1.6	2.5	3.3	4.2	5.0	6.6	8.4
“ remaining	4.2	0	0	0	0	0	0	0	0.1	0.5
Oxygen uptake		6.4	22.5	26.5	29.5	33.1	33.6	34.5	37.1	37.1
Phosphate uptake	0	2.4	8.2	10.8	12.4	15.8	17.4	19.6	21.4	22.9
Acid-labile phosphate	1.3	3.3	8.2	10.8	12.2	15.8	17.4	18.8	21.6	23.6
Barium salt insoluble at pH 7										
Acid-labile phosphate	0.4		5.9	6.3	7.9	8.3	8.4	8.5	7.9	8.3
Acid-stable “	0.3		0.3	0.4	0.7	0.9	1.2	0.5	1.1	1.6
N-R†	0	0	0	0	0	0	0	0	0	0
Barium salt soluble at pH 8.5										
Acid-labile phosphate			0.1	2.2	3.0	2.9	3.1	3.7	4.7	5.8
Acid-stable “			2.6	3.2	4.3	4.9	7.5	8.2	8.5	13.3
N-R†	3.0	0	0.7	1.6	2.6	3.8	4.8	5.4	7.6	9.0

* The whole vertical column represents an unincubated control, fixed at zero time.

† Nicotinamide-ribose moiety.

Purification of Nicotinamide Mononucleotide (NMN)—The procedure for separation of NMN from the other products of DPN splitting involved (1) deproteinization with trichloroacetic acid, (2) removal of barium salts insoluble at pH 8.4 (ATP, adenosine diphosphate, inorganic phosphate), (3) precipitation of the barium salt of NMN with acetone, (4) removal of compounds insoluble as mercury salts or complexes at pH 4 (DPN, adenylic acid), (5) adsorption of NMN on norit and elution with isoamyl alcohol.

Kidney particles in phosphate buffer (20 ml.) were incubated with DPN (400 micromoles) for 120 minutes at 37°. 25 ml. of cold 10 per cent trichloroacetic acid was added, and the resultant precipitate washed with cold 10 per cent trichloroacetic acid. The combined supernatant fluid and washings were neutralized, saturated barium acetate was added in excess, and the pH adjusted with NaOH to 8.4. The precipitate was washed with water and discarded. On addition of 500 ml. of cold acetone to the combined supernatant fluid and washings (87 ml.), a white flocculent precipitate formed, which turned to a yellow oil. This was centrifuged, washed with acetone, dried *in vacuo*, and dissolved in dilute HCl. Approximately 80 per cent of the N-R originally present in the trichloroacetic acid extract was recovered.

TABLE III

Phosphate Balance and Separation of Reaction Products of DPN Splitting in Respiring System

The incubation mixture contained 15 ml. of kidney particles in phosphate buffer, 1500 micromoles of glutamate, and DPN in a final volume of 18 ml. The mixture was shaken in oxygen. The values are expressed in micromoles.

	Phosphate			Adenine*	Pentose	N-R	DPN
	Ortho-	Acid-labile	Acid-stable				
Incubation mixture, 0 min.†	539	0	170‡	85‡	170‡	85	85
" " 60 " †	293	249					3
Barium salt, insoluble at pH 6.9							
Total insoluble barium salt	14	112		30		0	
Cadmium salt, insoluble		50		4			
" " soluble		71	31	25	28		
Barium salt, insoluble at pH 6.9–8.4	236	105	53	45	57	1	
" " soluble at pH 8.4	26	10	97		83	85	3

* Determined by measuring the absorption at 260 mμ with a coefficient of 1.6×10^7 sq. cm. × mole^{-1}.

† Trichloroacetic acid filtrate.

‡ Calculated from DPN determination; all other values are determined.

Barium was removed with H_2SO_4 and the precipitate washed with water. Mercuric acetate was added until precipitation was complete (pH 3.80); analysis of the precipitate showed that the N-R lost in this step was almost entirely in the form of unsplit DPN. The supernatant fluid plus washings was freed of mercury with H_2S and shaken for 30 minutes with 2 gm. of norit (pH 2.0). Less than 1 per cent of the NMN was unadsorbed. The adsorbed NMN was eluted by shaking four times for 30 minutes with 10 ml. of 10 per cent isoamyl alcohol. The combined eluates, which contained

75 per cent of the N-R present before adsorption, were concentrated under reduced pressure to 8 ml.

Analysis of this purified sample gave the following values in micromoles per ml.: N-R 12.6, pentose 12.4, total organic phosphate 14.5, acid-labile phosphate 0.8, orthophosphate 0.0, and DPN 0.04. The ultraviolet absorption spectrum revealed a peak at 265 mμ. With the molecular extinction coefficient for the nicotinamide nucleoside of Schlenk (17), a value of 15.4 micromoles per ml. was obtained. Reduction of the purified NMN with hydrosulfite resulted in a marked but unstable increase in absorption between 280 and 380 mμ, with a maximum at about 330 mμ.

TABLE IV

Relative Rates of Splitting of DPN and $DPNH_2$

The incubation mixtures contained 0.20 ml. of kidney particles in phosphate buffer, 0.75 ml. of water, and 0.05 ml. of either DPN or $DPNH_2$. The initial values for DPN were 2.71, 2.54, and 2.54 micromoles and for $DPNH_2$ 3.22, 2.63, and 2.73 micromoles for Experiments 1, 2, and 3, respectively. The vessels were gassed with nitrogen passed over heated copper. The vessel contents to be analyzed for DPN were fixed by tipping in 0.5 ml. of 20 per cent trichloroacetic acid from a side arm; those to be analyzed for $DPNH_2$ were fixed by plunging the vessel, still attached to the manometer, into boiling water. The values are expressed in micromoles.

Experiment No.	Incubation time	Amount split	
		DPN	$DPNH_2$
	min.		
1	15	0.83	1.25
2	15	0.17	0.65
3	15	0.47	1.19
	30	0.85	1.48

Relative Rates of Splitting of DPN and $DPNH_2$—It was observed that the rate of DPN disappearance was more rapid (1.4 to 1.8 times) in an actively respiring system (*i.e.* glutamate present) than in one to which no oxidizable substrate had been added. That this disappearance of DPN was a splitting and not an accumulation of the reduced form was indicated by the absence of $DPNH_2$ at the end of the incubation period.

It was considered that the accelerated disappearance of DPN in respiring systems might be due to its conversion to $DPNH_2$ and to a more rapid splitting of $DPNH_2$. Das and von Euler (4) have reported that the enzymatic release of orthophosphate was faster from $DPNH_2$ than from DPN. The data in Table IV, obtained in the absence of oxidizable substrate, show that $DPNH_2$ is destroyed more rapidly than DPN. Anaerobiosis was complete enough to prevent any significant reoxidation of $DPNH_2$. These data thus provide a possible explanation for the accelerated splitting of DPN in systems in which $DPNH_2$ is continuously being formed by substrate oxidation.

DPN Splitting by Rabbit Brain—In agreement with Handler and Klein (6), we have found splitting of DPN by brain more rapid than by kidney. While brain homogenate (0.5 ml. in an incubation mixture of 0.8 ml.) destroyed all but 0.8 of 8.9 micromoles of DPN after 30 minutes incubation, a similar concentration of kidney homogenate destroyed only 4.4 of 8.4 micromoles after 60 minutes incubation. Significant for the mechanism of DPN splitting by brain was the parallel disappearance of DPN and N-R (Table V). The rate of splitting of a purified preparation of NMN was even more rapid than that of DPN.

TABLE V

DPN Splitting by Rabbit Brain

The incubation mixtures were as follows: Experiment 1, 0.5 ml. of brain particles (in phosphate buffer), 0.15 ml. of neutralized DPN solution, 0.20 ml. of water; Experiment 2, 0.5 ml. of brain homogenate, 0.15 ml. of DPN, 0.10 ml. of water; Experiment 3, 1.0 ml. of brain particles (in bicarbonate buffer), 0.30 ml. of DPN, 0.20 ml. of water; Experiment 4, 10.0 ml. of brain particles (the same preparation as in Experiment 3), 0.30 ml. of crystalline muscle adenylic acid or purified nicotinamide mononucleotide, 0.20 ml. of water. The values are expressed in micromoles.

Experiment No.	Substance estimated	Values after incubation for			
		0 min.	15 min.	30 min.	60 min.
1	DPN	8.1	4.8	2.8	0.4
	N-R	8.1	4.9	2.6	0.5
2	DPN	8.9		0.3	
	N-R	8.9		0.3	
3	DPN	18.3	10.7	5.7	2.1
	N-R	18.3	12.2	7.4	2.4
4	NMN	12.3	3.0	1.1	0.7
	Adenylic acid	9.9	8.2	7.0	4.7

These preparations of brain particles also destroyed adenylic acid but at a relatively slow rate. Despite this slow rate of adenylic acid breakdown and the rapid splitting of DPN, adenylic acid could not be detected. In confirmation of Handler and Klein (6), no inorganic orthophosphate was detected during the splitting of DPN (nor was any inorganic orthophosphate found as a result of NMN splitting). Experiments with added adenylic acid revealed that adenylic acid disappearance agreed well, within 10 per cent, with release of inorganic orthophosphate.

Effects of Nicotinamide on DPN Splitting by Brain and Kidney—It has been reported (6, 18) that the inhibition by nicotinamide of DPN splitting by several tissues, including brain and kidney, is almost complete.

While these findings have been confirmed for brain, there was an almost complete lack of inhibitory effect by nicotinamide on DPN splitting by kidney particles (Table VI). The N-R values decreased at rates identical with DPN disappearance in experiments with brain, but, as stated in an earlier section, N-R values were almost unchanged during the course of DPN splitting by kidney. It is noteworthy that, while a slight decrease in N-R concentration was observed during DPN splitting by kidney in the absence of nicotinamide, there was no decrease when nicotinamide was present.

Table VI

Effects of Nicotinamide on DPN Splitting by Brain and Kidney

The incubation mixtures were as follows: Experiment 1, 0.30 ml. of brain (or kidney) particles in phosphate buffer ($MgCl_2$ and NaF omitted), 0.15 ml. of DPN, nicotinamide in the final concentrations shown, and water to a final volume of 0.55 ml. Experiment 2, 0.5 ml. of brain (or kidney) homogenate, 0.15 ml. of DPN, 0.08 ml. of 2 M nicotinamide, 0.07 ml. of water. Water replaced nicotinamide when the latter was omitted. The values are expressed in micromoles.

Tissue	Substance estimated	Experiment 1. Varying nicotinamide, 30 min. incubation					
		Nicotinamide					
		0.18 M*	0.18 M	0.09 M	0.045 M	0.018 M	0.000 M
Brain	DPN	7.7	8.2	7.7	7.7	6.2	3.5
	N-R	7.7	7.7	7.6	7.1	6.2	3.2
Kidney	DPN	7.4	5.4	5.6	5.6	5.9	5.9
	N-R	7.4	8.3	7.9	8.3	7.8	7.6
		Experiment 2. Varying time, ± 0.2 M nicotinamide					
		Time of incubation					
		0 min.	30 min.	60 min.	120 min.	180 min.	
Brain	Nicotinamide present, DPN	9.0	8.7	7.2			
	" " N-R	9.0	8.2	7.9			
"	" absent, DPN	8.9	0.3	0.2			
	" " N-R	8.9	0.3	0.3			
Kidney	" present, DPN	8.5		4.6	3.2	2.3	
	" " N-R	8.5		8.8	8.8	8.9	
"	" absent, DPN	8.4		4.0	3.1	2.1	
	" " N-R	8.4		7.3	7.2	7.2	

* The whole vertical column represents an unincubated control; fixed at zero time.

Evidence for DPN Nucleosidase in Kidney—The foregoing data indicate that the predominant mechanism of DPN splitting in rabbit kidney is by pyrophosphatase action. There is also evidence of a variable amount of nucleosidase activity. It has been noted that in eight of twenty preparations incubated until 50 per cent or more of the DPN was destroyed the N-R values decreased by more than 10 per cent. Further, it has been found that under conditions in which there was a decrease in N-R value during DPN splitting this decrease could be prevented by nicotinamide (Table VI, Experiment 2).

TABLE VII

Comparison of DPN Splitting by Kidney Homogenate, Residue, and Washed Particles

The preparation of kidney homogenate and particles (in phosphate buffer) was essentially as described under "Methods;" the residue was the sediment (not readily decanted) after centrifuging the original homogenate at 1500 R.P.M. for 5 minutes. Each fraction was adjusted to 15 ml. with phosphate buffer. The incubation mixtures were as follows: Experiments 1 and 2, 0.50 ml. of homogenate, 0.05 ml. of $MgCl_2$ (0.03 M), 0.05 ml. of NaF (0.1 M), 0.15 ml. of DPN; 1.0 ml. of residue, 0.10 ml. of $MgCl_2$ (0.03 M), 0.10 ml. of NaF (0.1 M), 0.30 ml. of DPN; 0.5 ml. of particles, 0.15 ml. of DPN, 0.1 ml. of water. The values are expressed in micromoles.

Experiment No.			Values after incubation for				
			0 min.	15 min.	30 min.	60 min.	120 min.
1	Homogenate	DPN	8.0				3.9
		N-R	8.0		7.2	7.5	7.5
	Residue	DPN	19.1				9.0
		N-R	19.1		19.6	21.8	21.8
	Particles	DPN	8.9				3.4
		N-R	8.9		8.9	8.9	9.6
2	Homogenate	DPN	8.0			4.0	
		N-R	8.0			9.2	
	Residue	DPN	14.4			9.9	
		N-R	14.4			15.5	
	Particles	DPN	8.1	6.4	5.7	4.5	
		N-R	8.1	9.2	9.5	10.0	

Since the absence of nucleosidase activity in some preparations of washed kidney particles might be due to its removal in the course of preparation, the activity of the unwashed homogenate and of the crude residue was tested. These crude kidney preparations, like the washed particles, contained no (or little) nucleosidase activity, as is shown in Table VII. It appears that the washed particles retained most of the DPN-splitting activity of the original homogenate.

DISCUSSION

The stoichiometric recovery of adenylic acid, the absence of significant changes in the concentrations of inorganic and organic phosphate, and the isolation of nicotinamide mononucleotide indicate that simple hydrolysis of the pyrophosphate bond is the most probable mechanism of DPN splitting by washed particles of rabbit kidney. In a system with active phosphate esterification, the data are consistent with this mechanism, since ATP is formed instead of adenylic acid.

The finding of a second pathway for enzymatic degradation of DPN and the fact that both pathways may occur in a single tissue may help clarify some questions which have arisen from assuming that DPNase is a single enzyme. Reliance on nicotinamide inhibition to preserve DPN or to evaluate DPN-splitting activity must be considered in the light of the specificity of this inhibition for nucleosidase. The failure of previous investigators to detect pyrophosphatase activity was probably related to methods of measurement. Handler and Klein (6) estimated DPN disappearance by loss of "factor V" activity for hemophilic bacteria, and Spaulding and Graham (19) relied only on the appearance of nicotinamide. Since Schlenk has shown that nicotinamide riboside satisfies "factor V" requirements (20), any splitting of DPN in which the nicotinamide-ribose moiety was preserved would not be detected by these methods.

SUMMARY

1. Two distinct enzymatic mechanisms exist for the degradation of DPN. Cleavage of the glycosidic bond between nicotinamide and the rest of the DPN molecule is catalyzed by "DPN nucleosidase." Cleavage of the pyrophosphate bond between adenylic acid and nicotinamide mononucleotide (NMN) is catalyzed by "DPN pyrophosphatase." NMN has been partially purified.

2. In rabbit brain, DPN splitting is by nucleosidase action. In rabbit kidney, pyrophosphatase action is the predominant mechanism. Nicotinamide inhibition is specific for nucleosidase.

3. Oxygen consumption and phosphate esterification by rabbit kidney particles are markedly stimulated by DPN. In a respiring system adenylic acid is recovered as adenosine triphosphate.

4. DPN disappearance is more rapid from a system in which substrate is being oxidized than from one to which no substrate has been added. Added $DPNH_2$ is split more rapidly than DPN under anaerobic conditions.

It is a pleasure to express our gratitude to Professor C. F. Cori for his constant guidance and encouragement.

BIBLIOGRAPHY

1. Schlenk, F., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, 5, 207 (1945).

2. Ohlmeyer, P., *Biochem. Z.*, **287**, 212 (1936).
3. Heiwinkel, H., *Ark. Kemi, Mineral. o. Geol.*, **13 A**, No. 19 (1939).
4. Das, N. B., and von Euler, H., *Nature*, **141**, 604 (1938).
5. Das, N. B., *Ark. Kemi, Mineral. o. Geol.*, **13 A**, No. 7 (1939).
6. Handler, P., and Klein, J. R., *J. Biol. Chem.*, **143**, 49 (1942).
7. Williamson, S., and Green, D. E., *J. Biol. Chem.*, **135**, 345 (1940).
8. Ohlmeyer, P., *Biochem. Z.*, **297**, 66 (1938).
9. Cori, G. T., Slein, M. W., and Cori, C. F., *J. Biol. Chem.*, **173**, 605 (1948).
10. Kubowitz, F., and Ott, P., *Biochem. Z.*, **314**, 94 (1943).
11. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **167**, 157 (1947).
12. Fiske, C., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
13. Cohn, G., and Kolthoff, I. M., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 886 (1942).
14. Mejbaum, W., *Z. physiol. Chem.*, **258**, 117 (1939).
15. Kalckar, H. M., *Science*, **99**, 131 (1944).
16. Cori, C. F., in A symposium on respiratory enzymes, Madison (1942).
17. Schlenk, F., *Arch. Biochem.*, **3**, 93 (1943).
18. Mann, P. J. G., and Quastel, J. H., *Biochem. J.*, **35**, 502 (1941).
19. Spaulding, M. E., and Graham, W. D., *J. Biol. Chem.*, **170**, 711 (1947).
20. Schlenk, F., in A symposium on respiratory enzymes, Madison (1942).

MICROBIOLOGICAL DETERMINATION OF PHENYLALANINE IN PROTEINS AND FOODS

By MILLARD J. HORN, D. BREESE JONES, AND AMOS E. BLUM

(From the Bureau of Human Nutrition and Home Economics, Agricultural Research Administration, United States Department of Agriculture, Washington)

(Received for publication, July 16, 1948)

Lactobacillus arabinosus (1–5), *Lactobacillus casei* (6–8), *Lactobacillus delbrueckii* LD5 (9), *Leuconostoc mesenteroides* P-60 (5, 10–12), and *Streptococcus faecalis* (5, 13, 14) have been either proposed or used for the assay of phenylalanine. In this work the basal medium used for methionine (15) had to be modified to obtain an adequate curve with *L. mesenteroides* P-60.

TABLE I

Composition of Basal Medium

	gm.		*mg.*
Glucose	20	DL-Alanine	80
Sodium acetate (anhydrous)	12	L-Arginine hydrochloride	416
Salts A		DL-Aspartic acid	240
K_2HPO_4	1	L-Cystine	400
KH_2PO_4	1	DL-Glutamic acid + H_2O	940
Salts B	*mg.*	Glycine	400
$MgSO_4 \cdot 7H_2O$	400	L-Histidine hydrochloride + H_2O	54
$MnSO_4 \cdot 4H_2O$	20	L-Hydroxyproline	20
NaCl	20	DL-Isoleucine	50
$FeSO_4 \cdot 7H_2O$	20	DL-Leucine	400
Adenine	100	DL-Lysine hydrochloride	300
Guanine	100	DL-Methionine	200
Uracil	100	DL-Norleucine	400
Thiamine chloride	2.0	DL-Phenylalanine*	160
Pyridoxamine dihydrochloride	0.4	L-Proline	140
Calcium pantothenate	0.4	DL-Serine	240
Riboflavin	0.4	DL-Threonine	180
Nicotinic acid	0.8	DL-Tryptophan	400
p-Aminobenzoic acid	0.4	L-Tyrosine	390
Biotin	0.01	DL-Valine	240
	γ		
Folic acid†	30‡		
Solution brought to 1000 cc. volume, pH 6.8			

* Omitted in phenylalanine determination.

† Obtained through the courtesy of Dr. R. J. Williams, The University of Texas.

‡ 30 γ of material of "potency 5000."

TABLE II

Recovery of Phenylalanine Added to Protein Hydrolysates

Protein hydrolysate	Phenylalanine				
	In hydrolysate*	Added	Total	Found	Recovery
	γ	γ	γ	γ	*per cent*
Barley, pearled	3.13	10.4	13.53	13.50	99
	6.25	10.4	16.65	16.75	101
	9.38	10.4	19.78	20.00	101
Egg, whole, dried	13.8	5.2	19.00	19.00	100
	13.8	10.4	24.20	24.50	101
	6.9	41.7	48.60	48.75	101
Ovalbumin	13.35	5.20	18.55	18.25	98
	13.35	10.40	23.75	24.00	101
	13.35	15.60	28.95	29.00	100
	6.68	41.70	48.38	48.00	99
Ox muscle	4.25	15.6	19.85	20.00	101
	8.50	15.6	24.10	24.50	102
	4.25	41.7	45.95	45.75	99

* Not corrected for moisture and ash.

TABLE III

*Phenylalanine Content of Some Proteins and Foods Determined at Different Assay Levels**

Assay level of material	Phenylalanine found									
	Ovalbumin		Brazil nut meal		Dry skim milk		Oatmeal		White rice	
γ	γ	*per cent*	γ	*per cent*	γ	*per cent*	γ	*per cent*	γ	*per cent*
100	6.70	6.70								
200	13.50	6.75	3.40	1.70	3.10	1.55				
300	20.50	6.83								
400	26.80	6.70	6.75	1.69	6.25	1.56				
500							3.50	0.70		
600			10.50	1.75	9.50	1.58				
800			13.80	1.73	13.00	1.62				
1000							7.0	0.70	3.30	0.33
1500							10.75	0.71		
2000							14.75	0.74	6.90	0.34
3000									10.30	0.34
4000									13.25	0.33
Average......		6.75		1.72		1.58		0.71		0.34

* Not corrected for moisture and ash.

The new basal medium (Table I) shows pyridoxine replaced by pyridoxamine (16), arginine increased from 96 to 416 mg. per liter (17), tyrosine increased 3-fold, norleucine increased from 120 to 400 mg., and L-methionine and L-tryptophan replaced by the DL compounds.

The requirement for extra tyrosine is interesting, as it has been shown by other workers (18–20) that there is a relationship between tyrosine and phenylalanine utilization.

EXPERIMENTAL

Leuconostoc mesenteroides P-60 was employed in the assays described.[1]

Basal Medium—The basal medium is shown in Table I.

TABLE IV

*Reproducibility of Phenylalanine Content When Determined by Separate Assays**

Material	Assay 1	Assay 2	Average
	per cent	*per cent*	*per cent*
Barley, pearled	0.63	0.61	0.62
Conarachin	4.00	4.10	4.05
Corn germ, defatted	0.79	0.79	0.79
" whole, yellow	0.63	0.61	0.62
Cottonseed flour	2.92	3.03	2.98
Edestin	5.14	5.00	5.07
Egg, whole, dried	3.45	3.24	3.35
Ovalbumin	6.68	6.75	6.72
Ox muscle	4.25	4.11	4.18
Peanut flour	3.13	2.81	2.97
Rice, white	0.34	0.32	0.33
Wheat germ, defatted	1.25	1.26	1.25
" whole	0.70	0.68	0.69
Yeast, dried, brewers'	1.67	1.50	1.58

* Not corrected for moisture and ash.

Assay Procedure—The procedures followed for the cultures, inoculum, and preparation of samples were identical with those described in other papers (15, 16).

Preparation of Phenylalanine Standards—L-Phenylalanine was used to prepare the standard curve (Fig. 1). The titration values on this curve were not altered by the addition to the medium of 1.2 mg. of any of the other nineteen amino acids.

Recovery of phenylalanine added to hydrolysates of barley, dried egg, ovalbumin, and ox muscle gave results well within the experimental error for this type of assay (Table II).

Table III shows values found for ovalbumin and several foods at differ-

---

[1] Obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D. C.

TABLE V

Phenylalanine Content of Some Proteins and Foods

Percentages calculated for ash and moisture-free material.

Material	N	Phenylalanine	Values from literature
	per cent	*per cent*	
Arachin	18.30	6.96	5.03 (21), 5.5 (22)
Casein	16.07	4.89	3.7 (2), 5.45 (5), 5.3 (8), 5.9 (9), 4.9 (11), 5.2 (12), 5.1 (13), 3.99 (21), 4.77 (23), 5.5 (24), 5.71 (25)
Coconut globulin	17.42	5.10	2.05 (26)
Conarachin	18.20	4.32	3.29 (21)
Cottonseed globulin	18.00	8.13	9.1 (27)
Edestin	18.55	5.43	4.2 (2), 5.45 (4), 5.22 (5)
Gelatin (Bacto)	18.32	2.33	2.2 (8), 2.3 (9), 1.57 (21), 2.24 (23), 2.45 (25)
Glycinin	17.30	5.82	
Lactalbumin	15.39	3.59	3.7 (8), 3.63 (21), 5.6 (22), 2.88 (23)
Ovalbumin (crystalline)	15.98	7.17	7.9 (9), 6.0 (22), 6.18 (25)
Ox muscle	16.00	4.58	3.9 (8), 4.5 (22), 4.92 (28)
Peanut, total globulins	18.01	5.75	
Phaseolin (navy bean)	16.07	8.04	
Wheat bran globulin	17.76	4.22	
Zein	16.00	7.30	7.1 (22), 6.77 (25)
Barley, pearled	1.86	0.68	0.45 (13)
Brazil nut meal	9.03	2.06	
Corn germ, defatted	3.93	0.96	1.37 (22), 0.71 (29)
" whole, yellow	2.22	0.73	0.74 (13), 0.62 (22)
Cottonseed flour	10.36	3.40	3.56 (13), 4.4 (22), 2.69 (29)
Egg, whole, dried	8.11	3.71	3.0 (22), 3.09 (29), 3.24 (30)
Milk, dry, skim	6.57	1.82	2.18 (9), 1.89 (12), 2.05 (13), 2.36 (22), 2.25 (31)
Oatmeal	2.73	0.79	0.82 (13), 1.18 (22)
Peanut flour	10.15	3.33	3.43 (22), 1.79 (29)
Peas, black-eyed	4.15	1.34	
Rice, white	1.26	0.38	0.49 (22)
Rye, whole	1.98	0.56	0.69 (9)
Soy bean flour	8.85	2.63	2.93 (9), 2.71 (13), 3.1 (22), 2.84 (29), 2.93 (32)
Wheat germ, defatted	6.50	1.41	1.7 (22), 1.66 (29)
" whole	3.07	0.78	0.96 (9), 0.81 (13), 1.07 (22)
Yeast, dried, brewers'	7.71	1.79	2.12 (9), 1.73 (29), 1.97 (33)

ent assay levels. Data on the reproducibility of values found for a number of materials when determined by separate assays are given in Table IV.

The results (Table V) found for the proteins and foods[2] agree quite well with other microbiological values, but do not agree with most of the values obtained by chemical methods. This is not surprising in view of difficulties inherent in the chemical methods for this amino acid.

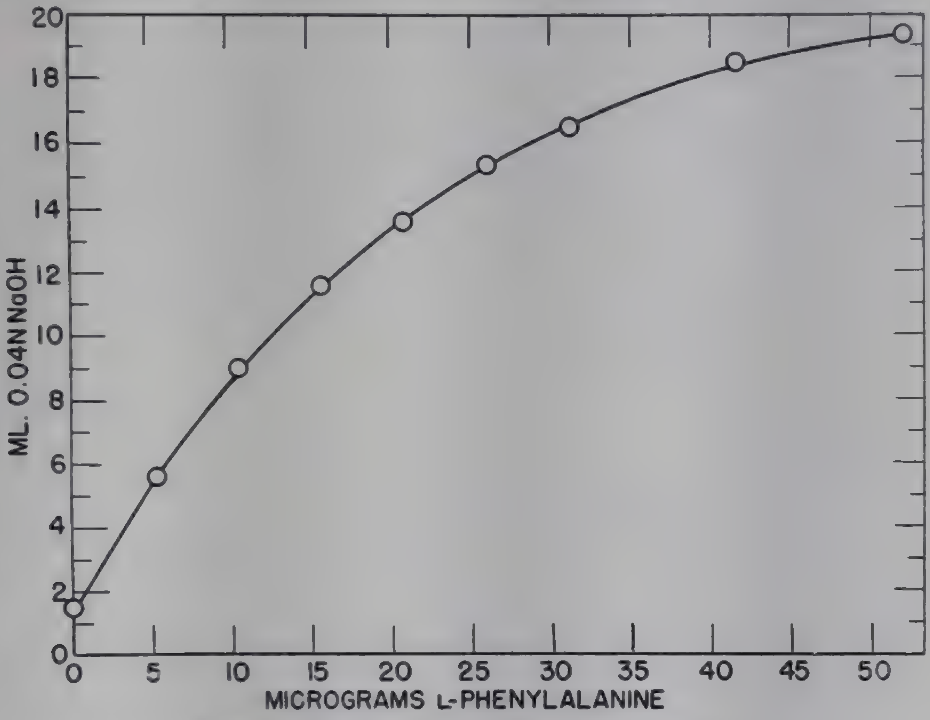


FIG. 1. Standard curve for phenylalanine

SUMMARY

A microbiological method is described for the determination of phenylalanine in proteins and foods with *Leuconostoc mesenteroides*. The results of assays on thirty-one proteins and foods agree closely with those obtained on the same materials by other microbiological methods.

BIBLIOGRAPHY

1. Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F., and Blotter, L., *J. Biol. Chem.*, **151**, 615 (1943).
2. Hegsted, D. M., *J. Biol. Chem.*, **152**, 193 (1944).
3. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, **166**, 417 (1946).
4. Smith, E. L., Greene, R. D., and Bartner, E., *J. Biol. Chem.*, **164**, 159 (1946).
5. Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172**, 15 (1948).
6. Hutchings, B. L., and Peterson, W. H., *Proc. Soc. Exp. Biol. and Med.*, **52**, 36 (1943).
7. Shankman, S., Dunn, M. S., and Rubin, L. B., *J. Biol. Chem.*, **151**, 511 (1943).

---

[2] The sources and preparations of the samples assayed are given in a previous publication on the determination of methionine (34).

8. Hier, S. W., Graham, C. E., Freides, R., and Klein, D., *J. Biol. Chem.*, **161**, 705 (1945).
9. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, **160**, 35 (1945).
10. Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, **156**, 703 (1944).
11. Dunn, M. S., Shankman, S., and Camien, M. N., *J. Biol. Chem.*, **161**, 643 (1945).
12. Hodson, A. Z., and Krueger, G. M., *Arch. Biochem.*, **10**, 55 (1946).
13. Baumgarten, W., Mather, A. N., and Stone, L., *Cereal Chem.*, **23**, 135 (1946).
14. Baumgarten, W., Stone, L., and Boruff, C. S., *Cereal Chem.*, **22**, 311 (1945).
15. Horn, M. J., Jones, D. B., and Blum, A. E., *J. Biol. Chem.*, **166**, 321 (1946).
16. Horn, M. J., Jones, D. B., and Blum, A. E., *J. Biol. Chem.*, **169**, 739 (1947).
17. Horn, M. J., Jones, D. B., and Blum, A. E., *J. Biol. Chem.*, **169**, 71 (1947).
18. Womack, M., and Rose, W. C., *J. Biol. Chem.*, **166**, 429 (1946).
19. Simmonds, S., Tatum, E. L., and Fruton, J. S., *J. Biol. Chem.*, **169**, 91 (1947).
20. Grau, C. R., *J. Biol. Chem.*, **170**, 661 (1947).
21. Brown, W. L., *J. Biol. Chem.*, **155**, 277 (1944).
22. Block, R. J., and Bolling, D., The amino acid composition of proteins and foods, Springfield (1945).
23. Albanese, A. A., *J. Biol. Chem.*, **155**, 291 (1944).
24. Williamson, M. B., *J. Biol. Chem.*, **156**, 47 (1944).
25. Hess, W. C., and Sullivan, M. X., *Arch. Biochem.*, **5**, 165 (1944).
26. Jones, D. B., and Johns, C. O., *J. Biol. Chem.*, **44**, 291 (1920).
27. Fontaine, T. D., Olcott, H. S., and Lowy, A., *Ind. and Eng. Chem.*, **34**, 116 (1942).
28. Beach, E. F., Munks, B., and Robinson, A., *J. Biol. Chem.*, **148**, 431 (1943).
29. Edwards, L. E., Sealock, R. R., O'Donnell, W. W., Bartlett, G. R., Barclay, M. B., Tully, R., Tybout, R. H., Box, J., and Murlin, J. R., *J. Nutr.*, **32**, 597 (1946).
30. Mitchell, H. H., and Block, R. J., *J. Biol. Chem.*, **163**, 599 (1946).
31. Hodson, A. Z., and Krueger, G. M., *Arch. Biochem.*, **12**, 51 (1947).
32. Riesen, W. H., Clandinin, D. R., Elvehjem, C. A., and Cravens, W. W., *J. Biol. Chem.*, **167**, 143 (1947).
33. Block, R. J., and Bolling, D., *Arch. Biochem.*, **7**, 313 (1945).
34. Horn, M. J., Jones, D. B., and Blum, A. E., *J. Biol. Chem.*, **166**, 313 (1946).

THE NUCLEOHISTONE OF BEEF SPLEEN

By MARY L. PETERMANN* and CATHERINE M. LAMB

(*From the Sloan-Kettering Institute for Cancer Research, New York*)

(Received for publication, July 21, 1948)

Nucleohistones, the principal nucleoprotein components of cell nuclei, are insoluble in the moderately dilute salt solutions (0.10 to 0.15 M) which are used to extract other tissue proteins. They may, however, be extracted from cell nuclei by NaCl solutions which are much stronger (1 M or more (1)) or much weaker (less than 0.02 M (2)). While the two types of extract are chemically similar, they differ markedly in physical properties, and the question arises as to which resembles most closely the "native" nucleohistone.

When nucleohistone is dissolved in M NaCl, it shows the high and anomalous viscosity and the birefringence of flow which are characteristic of free desoxypentose nucleic acid. That the nucleic acid and histone are largely dissociated in this solvent has been shown by several investigators (1, 3, 4). The water or dilute salt extracts on the other hand ordinarily do not show birefringence of flow, and usually have a low viscosity (1, 2). When purified preparations made from these water extracts have been characterized by sedimentation and diffusion methods, they have been found to possess a degree of molecular asymmetry which, although high for a protein, is far less than that found for free desoxypentose nucleic acid (5, 6). Here the nucleic acid and histone components appear to be much more tightly bound to each other than in strong salt solutions.

These striking differences have been attributed to three different factors. The first is the degree of dissociation of the nucleic acid-histone bond, which seems to depend upon the ionic strength of the solution. The second is the specific effect which certain ions such as iodide have on the viscosity of nucleic acid solutions (7). The third factor is the action of the enzyme desoxyribonuclease which is present in all these extracts, and which depolymerizes desoxypentose nucleic acid. This enzyme is inhibited in M NaCl but is very active in dilute salt solutions (1). Water extracts of nucleohistone made before this fact was realized have, therefore, probably suffered considerable degradation (2). In fact, even when the nucleohistone is extracted in M NaCl, some enzymatic degradation may take place while the fibers are being spun in 0.14 M NaCl, especially when the enzyme is present in high concentration, as it is in the spleen (8).

In order to obtain undegraded nucleohistone it is evidently necessary curves rise so steeply, the relative viscosity has been plotted on a logarithmic scale. The lower part of the viscosity-concentration curve given by Mirsky and Pollister (1) for thymus nucleohistone in M NaCl has been reproduced here for comparison. These highly viscous nucleohistone preparations also showed a strong birefringence of flow. The ability of citrate to inhibit desoxyribonuclease has also been utilized by Chargaff and Zamenhof (14) for the preparation of highly polymerized desoxypentose nucleic acid from yeast.

---

* Finney-Howell Research Foundation Fellow, 1947-48.

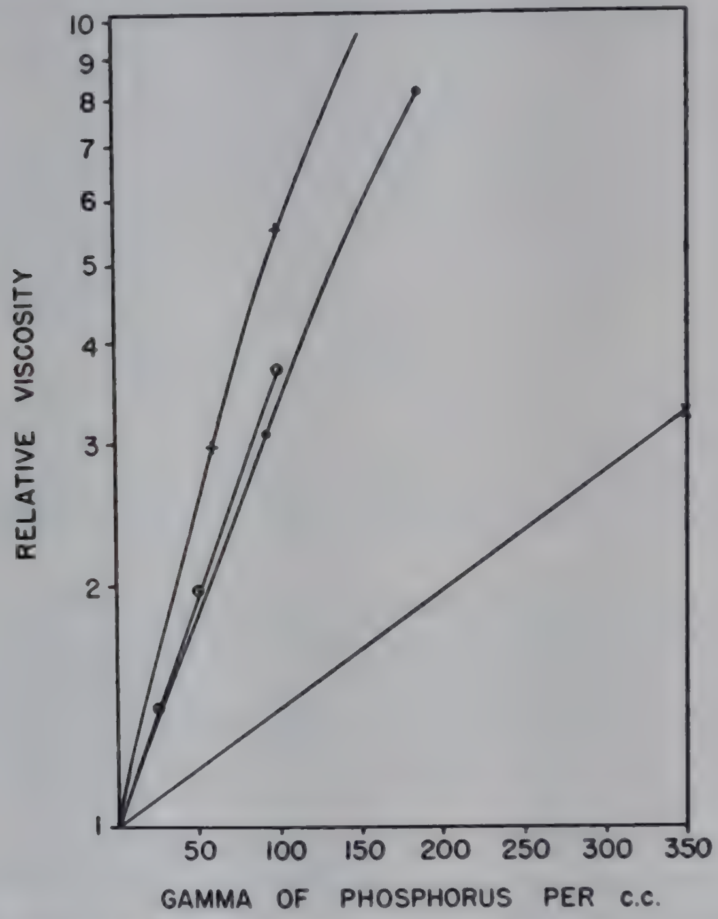


FIG. 1. The viscosity of beef spleen nucleohistone preparations made in the presence of varying amounts of citrate. All solutions contained M NaCl. × no citrate, ○ 0.05 M citrate, ● 0.01 M citrate, + thymus nucleohistone, no citrate (from Mirsky and Pollister (1)).

Upon dialysis against 0.01 M sodium citrate the nucleohistone precipitated in fibrous form, then redissolved completely to give a solution of unchanged nitrogen to phosphorus ratio. This solution had a viscosity almost as high as that found in M NaCl (see Fig. 2), and showed an equally strong flow birefringence. When 0.2 volume of 5.0 M NaCl was added, the nucleohistone precipitated as fibers, then redissolved to give a solution of the same viscosity as that of a 0.001 M citrate solution of the same concentration. When this solution was allowed to stand in the cold for 1 or more days, its viscosity (measured at 25°) increased somewhat, as shown in Fig. 2.

When these preparations were dialyzed against veronal buffer for electrophoretic analysis, they precipitated and dissolved in the same way. In one experiment, made on a 0.3 per cent solution in a buffer containing 0.02 M veronal, 0.01 M NaOH, and 0.01 M NaF, at pH 7.5, a mobility of −14 × 10^{-5} sq. cm. per sec. per volt was found. A second experiment was made on a 0.15 per cent solution in a buffer containing 0.02 M veronal, 0.01 M NaOH, and 0.001 M sodium citrate, at pH 7.5; here the mobility was −20.1 × 10^{-5}. In both experiments only one boundary was seen, but since the extremely high viscosity of these solutions could prevent the separation of components of different mobility, this cannot be considered evidence of homogeneity.

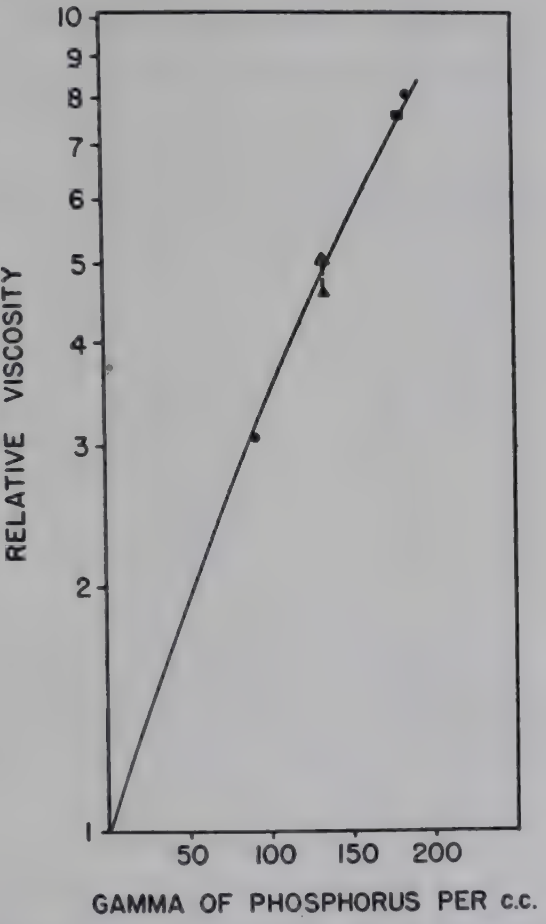


FIG. 2. The effect of the removal of sodium chloride on the viscosity of beef spleen nucleohistone. ● M NaCl + 0.01 M citrate, ■ 0.001 M citrate, ▲ 0.001 M citrate + M NaCl, △ 0.001 M citrate + M NaCl, 7 days later.

When the nucleohistone was centrifuged at 180,000 × *g* in 1.0 M NaCl plus 0.01 M citrate about 20 per cent of the protein nitrogen remained in the upper layers, while the phosphorus was almost completely removed (see Table I). The ultraviolet absorption curves obtained on the top two layers were characteristic of protein, with maxima at 275 and minima at 250 $m\mu$. The lower layers were extremely viscous; their nitrogen to phosphorus ratios were somewhat higher than that of the original nucleohistone and their ultraviolet absorption curves also indicated a slight excess of protein. In the pellets at the bottom of the tubes, on the other hand, both chemical analysis and ultraviolet absorption indicated a greater concentration of nucleic acid over histone. These results are similar to those of Mirsky and Pollister (1), who found that when thymus nucleohistone is centrifuged in M NaCl the nucleic acid sediments more rapidly than the histone. The dialysis studies of Cohen (3) and the ultracentrifugal data of Stern and Davis (4) provide further evidence that histone and nucleic acid are partially dissociated in strong salt solutions.

TABLE I

Sedimentation of Spleen Nucleohistone

Solvent	Fraction	N		P		N:P ratio		Ultraviolet absorption curve
		Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	
		γ per cc.	γ per cc.	γ per cc.	γ per cc.			
M NaCl + 0.01 M citrate	Original	865	703	255	185	3.39	3.80	Typical nucleohistone
	0–2 cc.	73	87	1.1	1.3	66	67	Histone
	2–4 "	106	129	2.5	3.4	42	38	"
	4–6 "	486	365	120	78	4.05	4.67	Nucleohistone
	6 cc. to gel		872		186		4.69	"
	Pellet		3522		1125		3.13	Between nucleohistone and nucleic acid
		Experiment 3	Experiment 4	Experiment 3	Experiment 4	Experiment 3	Experiment 4	
0.001 M citrate	Original	397	664	106	180	3.74	3.69	Nucleohistone
	0–2 cc.	7*	4.3*	1	1.2	7*	3.6*	" and extra histone
	2–4 "	10	8.7	2	1.6	5	5.4	" "
	4–6 "†		857		250		3.42	Nucleohistone
	6–7 "†	1270	2548	346	628	3.66	4.06	"

* Values too small for accurate measurement.

† Gel.

In 0.001 M citrate, on the other hand, the histone was sedimented with the nucleic acid; only traces of either remained in the upper layers. The lower layers had nitrogen to phosphorus ratios and ultraviolet absorption curves very like those of the original nucleohistone solution (see Table I). These results demonstrated that in 0.001 M citrate solution the nucleic acid and histone were not appreciably dissociated. Since these nucleohistone preparations showed the same high viscosity and flow birefringence as the solutions in M NaCl and had retained their ability to precipitate in fibrous form, they must still have been composed of highly asymmetrical particles. Nucleohistone can, therefore, show the properties of a linear molecule even when the nucleic acid and histone are firmly bound.

In both the M NaCl and the dilute citrate experiments the residual chromosomes described by Mirsky and Ris (15) formed opaque pellets at the bottom of the centrifuge tubes. In Experiment 4, in 0.001 M citrate, the bottom layer was found to have a higher nitrogen to phosphorus ratio than the original nucleohistone (see Table I). This is in agreement with Mirsky's finding that the residual chromosomes have a lower nucleic acid content than the nucleohistone.

The nucleohistone in the intermediate layers, from which the residual chromosomes had been removed, had a nitrogen to phosphorus ratio of 3.4, somewhat lower than that of the original extract. This appears to be the best value for the nitrogen to phosphorus ratio of nucleohistone free of residual chromosomes. The nitrogen to phosphorus ratios obtained on the total nucleohistone extracts (after several reprecipitations) varied from 3.9 to 3.4. This variation probably means that different amounts of residual chromosomes were carried through the nucleohistone isolations.

The nucleic acid was prepared from spleen nucleohistone in the form of the sodium salt. Its ultraviolet absorption curve was similar to that of a thymus nucleate prepared by a modification[1] of the method of Gulland, Jordan, and Threlfall (16). The spleen nucleate contained 9.3 per cent phosphorus and 15.1 per cent nitrogen; the theoretical values are 9.4 and 15.9 per cent for sodium nucleate containing adenine, guanine, thymine, and cytosine in equal amounts (16). A low nitrogen content, 15.5 per cent, was also found by Gulland *et al.* in their thymus nucleate (16). The concentration of purine-bound desoxypentose was 105 per cent that of a thymus nucleic acid preparation of the same phosphorus content. The nucleate also contained about 2 per cent of pentose nucleic acid, an amount similar to that found by von Euler and Hahn in nucleic acid preparations from thymus and liver nuclei (13). In this laboratory small amounts of pentose have been found in thymus nucleates prepared by either the Hammarsten (10) or Gulland (16) procedures.

[1] Petermann, M. L., and Mason, E. J., unpublished experiments.

The nitrogen to phosphorus ratio of the sodium nucleate was 1.63, while that of the nucleohistone free of residual chromosomes was 3.42. The nucleohistone therefore contained close to 50 per cent of histone.

Whether the treatment with strong sodium chloride and with citrate has caused any irreversible changes in the nucleohistone molecule is not yet known. It has been claimed that the fibrous nature of nucleohistone in M NaCl is evidence of denaturation and that the "native nucleoprotein" isolated with the use of arsenate as an enzyme inhibitor is a relatively symmetrical molecule (6). In an attempt to answer this question we have begun the study of nucleohistone extracted from nuclei by water instead of by M NaCl.

When nucleohistone is extracted from spleen nuclei by water, the extracts are unstable even in the presence of citrate. Since the thymus contains much less desoxyribonuclease than the spleen, it was thought advisable to study water extracts of thymus nuclei washed with 0.05 M citrate. These extracts have a viscosity as high as that found in M NaCl. They do not exhibit the strong flow birefringence which is found with the strong salt extracts, but do show the "silkiness" characteristic of asymmetrical particles in suspension. When the salt concentration is increased to 0.15 M, the nucleohistone precipitates as fibers which have the spontaneous birefringence described by Cohen.[2] On repeated precipitation some preparations retain their high viscosity in water solution and their ability to precipitate as fibers, while others do not. Ahlström, von Euler, and Hahn (17) have also obtained highly viscous water extracts of nucleohistone, from thymus nuclei isolated in citric acid. The results of these experiments are far from conclusive; they do, however, suggest that the native nucleoprotein is present in the nucleus in a far more asymmetrical form than that found for the arsenate preparations (6).

SUMMARY

1. Nucleohistone preparations of high viscosity can be prepared from beef spleen by extraction with M NaCl in the presence of 0.01 M sodium citrate.

2. On dialysis against 0.001 M citrate the nucleohistone precipitates as fibers, then redissolves to give a solution of only slightly decreased viscosity, which still shows birefringence of flow. High speed centrifugation experiments show that in this solvent the nucleic acid and histone are tightly linked. Nucleohistone can, therefore, show the properties of a linear molecule even when the nucleic acid and histone are firmly bound.

3. Sodium nucleate prepared from the spleen nucleohistone resembled

---

[2] Cohen, S. S., personal communication.

thymus nucleate in its ultraviolet absorption characteristics and in its content of nitrogen, phosphorus, purine-bound desoxypentose, and pentose.

The authors gratefully acknowledge the cooperation of Sidney S. Furst in carrying out the high speed centrifugation experiments. Some of the expenses of this investigation were defrayed by a grant from the James Foundation of New York, Inc.

BIBLIOGRAPHY

1. Mirsky, A. E., and Pollister, A. W., *J. Gen. Physiol.*, **30**, 117 (1946).
2. Carter, R. O., and Hall, J. L., *J. Am. Chem. Soc.*, **62**, 1194 (1940).
3. Cohen, S. S., *J. Biol. Chem.*, **158**, 255 (1945).
4. Stern, K. G., and Davis, S., *Federation Proc.*, **5**, 156 (1946).
5. Carter, R. O., *J. Am. Chem. Soc.*, **63**, 1960 (1941).
6. Stern, K. G., Goldstein, G., Wagman, J., and Schryver, J., *Federation Proc.*, **6**, 296 (1947).
7. Greenstein, J. P., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **9**, 236 (1941).
8. Greenstein, J. P., *J. Nat. Cancer Inst.*, **2**, 357 (1941).
9. McCarty, M., *J. Gen. Physiol.*, **29**, 123 (1946).
10. Hammarsten, E., *Biochem. Z.*, **144**, 387 (1924).
11. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
12. Dische, Z., *Mikrochemie*, **8**, 4 (1930).
13. von Euler, H., and Hahn, L., *Svensk Kem. Tidskr.*, **58**, 251 (1946).
14. Chargaff, E., and Zamenhof, S., *J. Biol. Chem.*, **173**, 327 (1948).
15. Mirsky, A. E., and Ris, H., *J. Gen. Physiol.*, **31**, 1 (1947).
16. Gulland, J. M., Jordan, D. O., and Threlfall, C. J., *J. Chem. Soc.*, 1129 (1947).
17. Ahlström, L., von Euler, H., and Hahn, L., *Ark. Kemi, Mineral. o. Geol.*, **22 A**, No. 13 (1946).

THE APPLICATION OF STRUCTURAL ANALOGUES TO ENZYMATIC STUDIES*

I. STUDIES ON THE MODE OF ACTION OF BIOTIN ANALOGUES

BY A. E. AXELROD, SARAH ELLEN PURVIS, AND KLAUS HOFMANN

(From the Institute of Pathology, Western Pennsylvania Hospital, and the Department of Chemistry, University of Pittsburgh, Pittsburgh)

(Received for publication, June 30, 1948)

In recent communications (1, 2) the synthesis of a number of biotin analogues was described, and their antagonistic effects towards biotin and oxybiotin for a variety of microorganisms have been presented. Since it seems reasonable to assume that biotin functions as a constituent of an essential enzyme system, the growth-inhibiting properties of the antagonists may be due to their interference with either the biosynthesis or functioning of this system. In 1944, Winzler, Burk, and du Vigneaud (3) observed a pronounced stimulatory effect of biotin upon the fermentation rate of biotin-deficient yeast. It appeared to us that their test system would be well suited for further studies of the mode of action of our antagonists. These compounds were found to counteract the stimulatory effects upon the fermentation rate of yeast of both biotin and oxybiotin. The inhibitory effects were noted only when the inhibitors were added *prior* to the vitamin. This indicated that the antagonists function by preventing the fixation of biotin to certain cellular constituents and are without effect upon the enzymatically active form ("bound" biotin).

Methods

The experimental procedures employed in this study were patterned after those of Winzler, Burk, and du Vigneaud (3).

The basal medium employed for the growth of the yeast *Saccharomyces cerevisiae* 139 was the Hertz modification (4) of the medium described by Snell, Eakin, and Williams (5), with the omission of the casein hydrolysate. Growth on this medium was negligible but could be stimulated by either biotin or oxybiotin. The "high" biotin and oxybiotin media contained 5 mγ (millimicrograms) of biotin and 10 mγ of oxybiotin per 10 ml., respectively, and yielded maximum growth. Poor growth was obtained on the "low" biotin and oxybiotin media which contained 0.08 mγ of biotin and 0.5 mγ of oxybiotin per 10 ml., respectively. 60 ml. portions of the various media were sterilized by autoclaving at 15 pounds for 10 minutes.

---

* Supported in part by grants from the American Cancer Society recommended by the Committee on Growth of the National Research Council.

After cooling, each flask was inoculated with a saline suspension of yeast grown for 24 hours of wort agar slants. After 20 hours incubation at 30°, the resulting yeast suspension was centrifuged and washed twice with the salt solution[1] used in the growth medium. The yeast was finally resuspended in the salt solution and suitable aliquots taken for the metabolic studies. The yeast concentration of these aliquots was determined by turbidity measurements in a Lumetron photocolorimeter and a calibration curve relating turbidity reading to dry weight.

Respiration (oxygen consumption) and fermentation (carbon dioxide production) measurements were made at 30° with constant volume Warburg manometers. The respiration rates were determined with air as the gas phase and 0.2 ml. of 10 per cent potassium hydroxide in the inner well. Gaseous phases of air and purified nitrogen were used for the aerobic and anaerobic measurements of fermentation, respectively. The conclusions drawn from the measurements under aerobic and anaerobic conditions were essentially the same. Since the majority of the fermentation studies were performed aerobically, only the aerobic fermentation rates will be presented. The oxygen uptake was usually neglected in calculating the aerobic fermentation rates, since it was small compared to the carbon dioxide evolution. The following constituents were always present in each Warburg flask: 0.5 ml. of the yeast suspension in the salt solution,[2] 0.5 ml. of the salt solution, and 40 mg. of glucose. The final liquid volume per flask was adjusted to 2.0 ml. with solutions of biotin, oxybiotin, aspartic acid, and inhibitors as indicated in the text. All experiments were conducted at 30° and pH 4.0. At the conclusion of each experimental run, the turbidity of the flask contents was determined. Cell counts were also occasionally performed. In no case was any significant amount of growth obtained.

EXPERIMENTAL

Effects of Biotin and Oxybiotin on Yeast Metabolism—It has been reported (3) that the fermentation and respiration rates of a biotin-deficient yeast are considerably lower than those of a normal yeast. We have confirmed these observations and, in addition, have shown that these metabolic processes were also markedly lowered in an oxybiotin-deficient yeast, *i.e.*, yeast grown in the presence of suboptimum amounts of *dl*-oxybiotin (0.5 mγ per 10 ml). These results are presented in Table I.

---

[1] Throughout this study the concentration of the salt solution added to the Warburg flasks was twice that used in the growth medium. The pH of this solution was always adjusted to 4.0 before use. Each ml. contained 5 mg. of ammonium sulfate.

[2] Amounts of dry yeast ranging from 0.5 to 5.0 mg. per flask were employed.

The "biotin effect," *i.e.* the stimulatory effect of biotin upon the fermentation rate of a biotin-deficient yeast (3), has also been observed in this laboratory. Oxybiotin could elicit a similar increase in an oxybiotin-deficient yeast, as illustrated in Fig. 1 ("oxybiotin effect"). In this respect biotin was more effective than oxybiotin. A similar situation exists with regard to the growth-stimulating properties of these two compounds for yeast, oxybiotin having only 25 per cent of the growth-promoting potency of biotin. The fermentation-stimulating activity of oxybiotin

TABLE I

*Respiration and Fermentation Rates of "High" and "Low" Biotin and Oxybiotin Yeasts**

	Low biotin	High biotin	Low oxybiotin	High oxybiotin
$-Q_{O_2}$	18	61	18	48
Q_{CO_2}	140	370	194	376

* The yeasts were grown as described in the previous section. The symbols $-Q_{O_2}$ and Q_{CO_2} represent respiration and aerobic fermentation, respectively, expressed in c.mm. per hour per mg. of dry weight. The Q values were constant during the experimental period of 2 hours. Each Warburg flask contained yeast, salt solution, and glucose as indicated in "Methods." All constituents were combined before temperature equilibration.

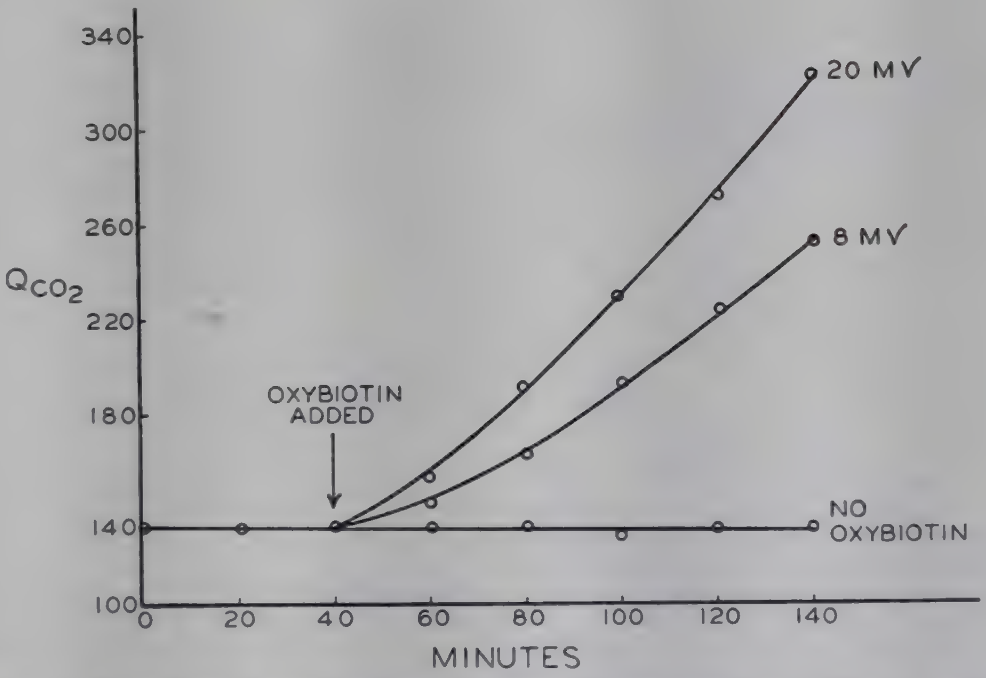
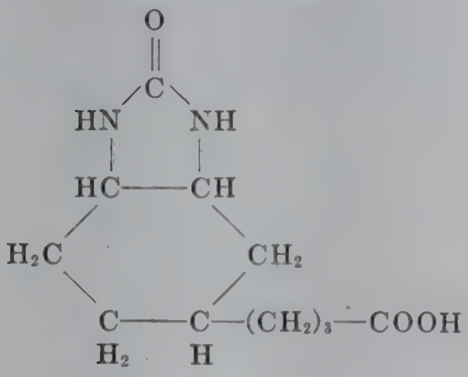


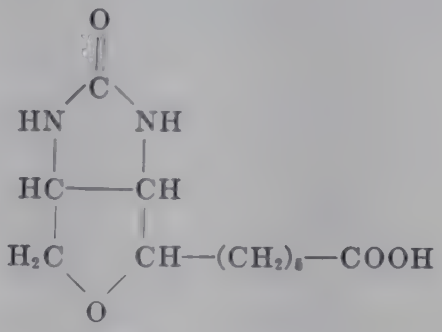
FIG. 1. The effect of oxybiotin on the aerobic fermentation of "low" oxybiotin yeast. 1.7 mg. of yeast per flask; oxybiotin added as indicated; other conditions identical with those described in Table I.

was also approximately 25 per cent that of biotin. It appears, therefore, that the effects upon growth correlate well with the metabolic effects, thus emphasizing the significance of the enzymatic rôle of biotin and oxybiotin in the growth process. As previously noted for the "biotin effect" (3), the stimulation by oxybiotin could be obtained only in the presence of ammonium sulfate.

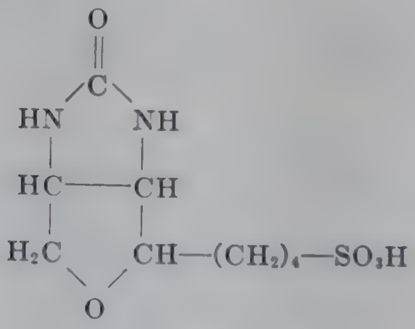
Effect of Structural Analogues of Biotin on Metabolic Processes of Yeast—The three compounds depicted have been shown to inhibit the growth-promoting properties of both biotin and oxybiotin. Their ability to counteract the above "biotin and oxybiotin effects" on fermentation have now been investigated. The antagonistic activity of oxybiotinsulfonic acid is illustrated in Fig. 2.



γ-(3,4-Ureylenecyclohexyl)-butyric acid[3]



Homooxybiotin



Oxybiotinsulfonic acid

It may be seen that the basal fermentation rate was not affected by $500\ \gamma$ of the inhibitor (Curve I). However, the stimulatory effect of $20\ m\gamma$ of oxybiotin (Curve III) could be completely abolished by $500\ \gamma$ of the inhibitor added *prior* to the oxybiotin (Curve I). The stimulatory effect of biotin could also be inhibited by this analogue. The behavior of the other

---

[3] We are indebted to Dr. J. P. English of the American Cyanamid Company for the γ-(3,4-ureylenecyclohexyl)-butyric acid.

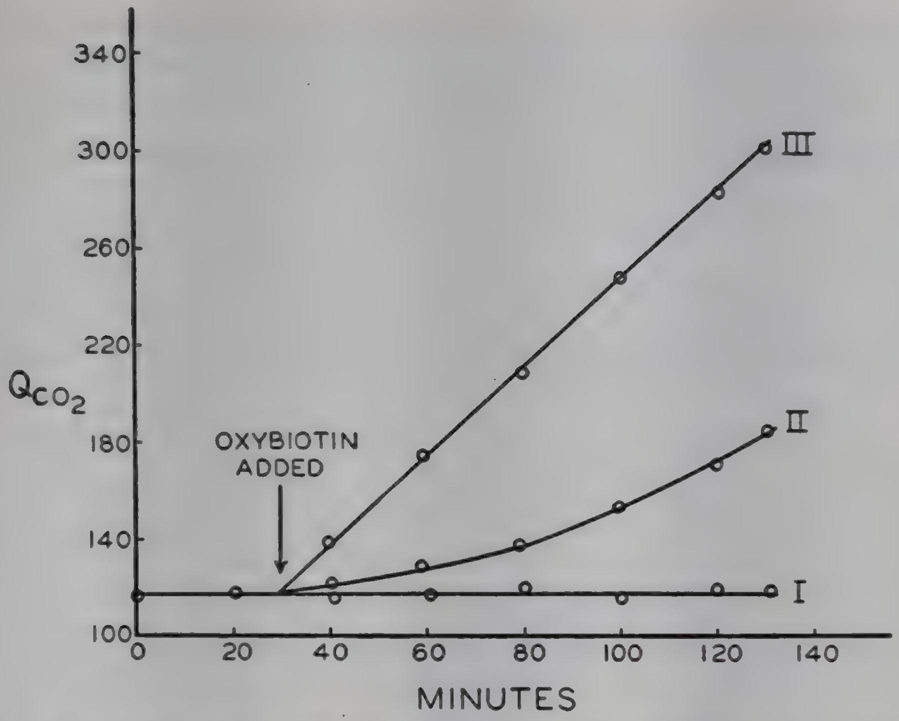


FIG. 2. Inhibition of the fermentation-stimulatory effect of oxybiotin by oxybiotinsulfonic acid. Each flask contained 1.8 mg. of "low" oxybiotin yeast; oxybiotinsulfonic acid added before temperature equilibration; other conditions as described in Table I. Curve I, fermentation rate under three different experimental conditions: (1) no addition, (2) 500 γ of inhibitor, and (3) 20 mγ of oxybiotin + 500 γ of inhibitor; Curve II, 20 mγ of oxybiotin + 20 γ of inhibitor; Curve III, 20 mγ of oxybiotin.

TABLE II

Inhibitory Effects of Biotin Analogues on Stimulation of Yeast Fermentation and Growth Due to Biotin and Oxybiotin

Biotin analogue	Inhibition ratio*			
	Biotin		Oxybiotin	
	Fermentation	Growth†	Fermentation	Growth†
γ-(3,4-Ureylenecyclohexyl)-butyric acid	1,000,000		75,000	
Homooxybiotin	375,000	445,000	3,750	7,400
Oxybiotinsulfonic acid	600,000	1,460,000	5,000	16,600

* Millimicrograms of inhibitor necessary to abolish completely the effect of 1 mγ of biotin or oxybiotin upon either fermentation or growth.

† Unpublished observations (Axelrod and Hofmann).

two compounds was found to be similar to that of oxybiotinsulfonic acid. The effects of the three inhibitors upon the fermentation-stimulating activities of both biotin and oxybiotin are shown in Table II. The conditions of these experiments were identical with those described in Fig. 2. Varying quantities of the inhibitors were added before temperature equilibration. Biotin (1 mγ) and oxybiotin (20 mγ) were added 30 minutes *after* the zero reading. In this manner, the minimum amount of inhibitor necessary to nullify the stimulatory effects was determined. For comparison, the growth-inhibiting potency of the analogues is also reported in Table II. It is apparent that the inhibition ratios for oxybiotin are considerably smaller than those for biotin. This indicates that both the fermentation and growth-stimulating activities of oxybiotin are more easily counteracted than those of biotin. The above correlation between metabolic effect and growth is again evident. The competitive nature of these inhibitions is indicated by the constancy of the inhibition ratios both for growth and fermentation over a wide range of biotin or oxybiotin concentrations.

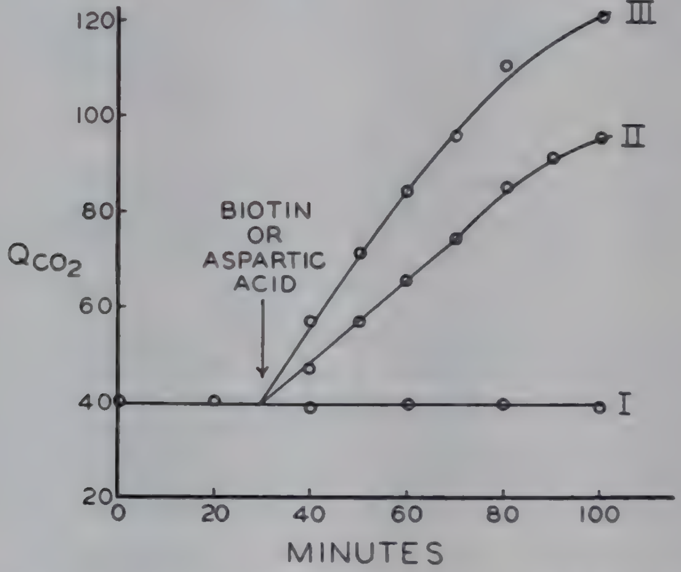


FIG. 3. Effect of aspartic acid on the fermentation of a biotin-deficient yeast. Each flask contained 3.9 mg. of yeast; biotin (5 mγ) and L-aspartic acid (800 γ) added as indicated; 7 mg. of γ-(3,4-ureylenecyclohexyl)-butyric acid added before temperature equilibration; other conditions as described in Table I. Curve I, fermentation rate under two different conditions, (1) no additions, (2) biotin + inhibitor; Curve II, fermentation rate under two different conditions, (1) aspartic acid, (2) aspartic acid + inhibitor; Curve III, biotin.

In another set of experiments, the inhibitors were added 20 minutes *after* the biotin or oxybiotin. Under these conditions none of the inhibitors was able to prevent the progressive increase in the fermentation rates.

Stimulatory Activity of L-*Aspartic Acid*—As seen in Fig. 3, L-aspartic acid was capable of stimulating the fermentation of a biotin-deficient yeast in the absence of added biotin (Curve II). In the absence of ammonia, the ability of biotin to stimulate fermentation was markedly curtailed; however, the stimulatory activity of aspartic acid was not affected. Similar results have been obtained by Winzler *et al.* (3). Of interest was the observation that amounts of inhibitor which were capable of completely suppressing the activity of biotin were without effect upon the stimulation by aspartic acid (Curves I and II).

DISCUSSION

In recent years many structural analogues of vitamins have been prepared and their ability to antagonize the growth-promoting effects of the parent compound has been fully investigated. It has been frequently suggested that the analogues might serve as effective tools in enzymatic studies relating to the mode of action of the vitamins. The experiments herein reported represent such a study and furnish some insight into the mechanism by which biotin analogues exert their growth-inhibiting effects. Since it is recognized that vitamins act as constituents of enzyme systems, the analogues could interfere with either the formation or the function of the active enzyme.

The observation that the antagonists counteract the "biotin effect" on fermentation only when added *prior* to the vitamin strongly indicates a successful competition with biotin in the biosynthesis of the enzymatically active complex ("bound" biotin). This complex may be regarded as a combination of either biotin or a biotin derivative with a specific protein. The inhibitors are unable to displace biotin or the biotin coenzyme from this combination and are incapable of interfering with its enzymatic function. This affords a logical explanation for the inability of the analogues to affect the basal fermentation rates of yeast where the limiting factor seems to be the amount of biotin-enzyme present in the cells. Experiments reported by Winzler *et al.* (3) are of interest in this connection. These authors observed that the "biotin effect" was more rapidly established when the yeast was incubated with biotin *prior* to the addition of ammonia. It seems likely that during this "pretreatment" the vitamin was converted into the enzyme essential for ammonia assimilation. The inhibition of the "biotin effect" by azide or cyanide and the failure of these compounds to inhibit the basal fermentation rate (3) could also be similarly explained. Thus the inhibitory activities of azide or cyanide might be due to their ability to inhibit certain energy-yielding processes which are necessary for the synthesis of the biotin-enzyme complex from biotin.

A relationship between biotin and aspartic acid has been indicated both for microbial growth (6, 7) and yeast fermentation (3). As shown by Winzler *et al.* (3) and confirmed in our experiments, aspartic acid is capable of stimulating yeast fermentation in the absence of both biotin and ammonia.

Of interest was our observation that the aspartic acid effect was not inhibited by the biotin analogues. Thus one of the functions of biotin could be related to its ability to catalyze the formation of aspartic acid in this system. The mode of action of aspartic acid in yeast fermentation remains to be elucidated.

SUMMARY

Studies on the mode of action of structural analogues of biotin have been conducted with both biotin and oxybiotin-deficient yeasts. The following observations have been made: (1) The fermentation rate of both biotin and oxybiotin-deficient yeasts was lower than that of normal yeast. This rate could be markedly accelerated by either biotin or oxybiotin. (2) A number of biotin analogues were capable of inhibiting the "biotin or oxybiotin effects" only when added *prior* to the vitamin. (3) The stimulatory effect of aspartic acid on yeast fermentation was not inhibited by the structural analogues.

It was proposed that the structural analogues exert their growth and fermentation-inhibiting effects by preventing the biosynthesis of an enzymatically active complex from biotin. One of the functions of this complex may be related to the synthesis of aspartic acid.

BIBLIOGRAPHY

1. Hofmann, K., Chen, C., Bridgwater, A., and Axelrod, A. E., *J. Am. Chem. Soc.*, **69**, 191 (1947).
2. Hofmann, K., Bridgwater, A., and Axelrod, A. E., *J. Am. Chem. Soc.*, **69**, 1550 (1947).
3. Winzler, R. J., Burk, D., and du Vigneaud, V., *Arch. Biochem.*, **5**, 25 (1944).
4. Hertz, R., *Proc. Soc. Exp. Biol. and Med.*, **52**, 15 (1943).
5. Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, **62**, 175 (1940).
6. Koser, S. A., Wright, M. H., and Dorfman, A., *Proc. Soc. Exp. Biol. and Med.*, **51**, 204 (1942).
7. Stokes, J. L., Larsen, A., and Gunness, M., *J. Bact.*, **54**, 219 (1947).

THE SEPARATION AND QUANTITATIVE ESTIMATION OF PURINES AND PYRIMIDINES IN MINUTE AMOUNTS*

By ERNST VISCHER† and ERWIN CHARGAFF

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, June 30, 1948)

The investigation of an entire series of compounds of great biological importance, the nucleic acids, nucleotides, and related substances, has been impeded considerably by the lack of specific methods for the characterization and estimation of their nitrogenous constituents. Many problems concerning the composition, metabolism, and biological functions of these substances cannot even be approached owing to the absence of sufficiently simple and widely applicable procedures.

The older methods for the determination of the total quantity of purines, reviewed by Jorpes (4), did not permit the identification of individual components. They were based, in the main, on the estimation of the nitrogen content of the purine mixtures precipitated with silver (5) or copper (6). A microprocedure founded on similar principles was described by Graff and Maculla (7). The introduction by Schmidt (8) of specific enzymes for analytical purposes represented a definite advance. More recently, Kalckar (9) made very elegant use of a combination of enzymatic and spectroscopic methods for the microestimation of individual purines. The attempt to determine the adenine content of purine mixtures by means of an adenine-deficient *Neurospora* mutant (10) has not yet given reliable results (11).

The pyrimidines have been neglected to an even higher degree. No specific methods seem to be available, and this has certainly handicapped our understanding of this important class of substances.

The procedure presented in this paper comprises essentially three steps: (*a*) the separation of the mixtures into individual components by means of chromatography on paper strips (12), (*b*) the demonstration of the number and position of separated compounds by their conversion into suitable metal salts, (*c*) the identification of the separated purines and pyrimidines through the shapes of their characteristic absorption curves in the ultraviolet and their quantitative estimation from the extinction values.

---

* This work has been supported by a research grant from the United States Public Health Service. Brief notes on some of its phases have appeared (1, 2); it was also discussed at the Cold Spring Harbor Symposium on Quantitative Biology, June, 1947, (3) and at the Fourth International Congress for Microbiology in Copenhagen, July, 1947.

† Swiss-American Student Exchange Fellow.

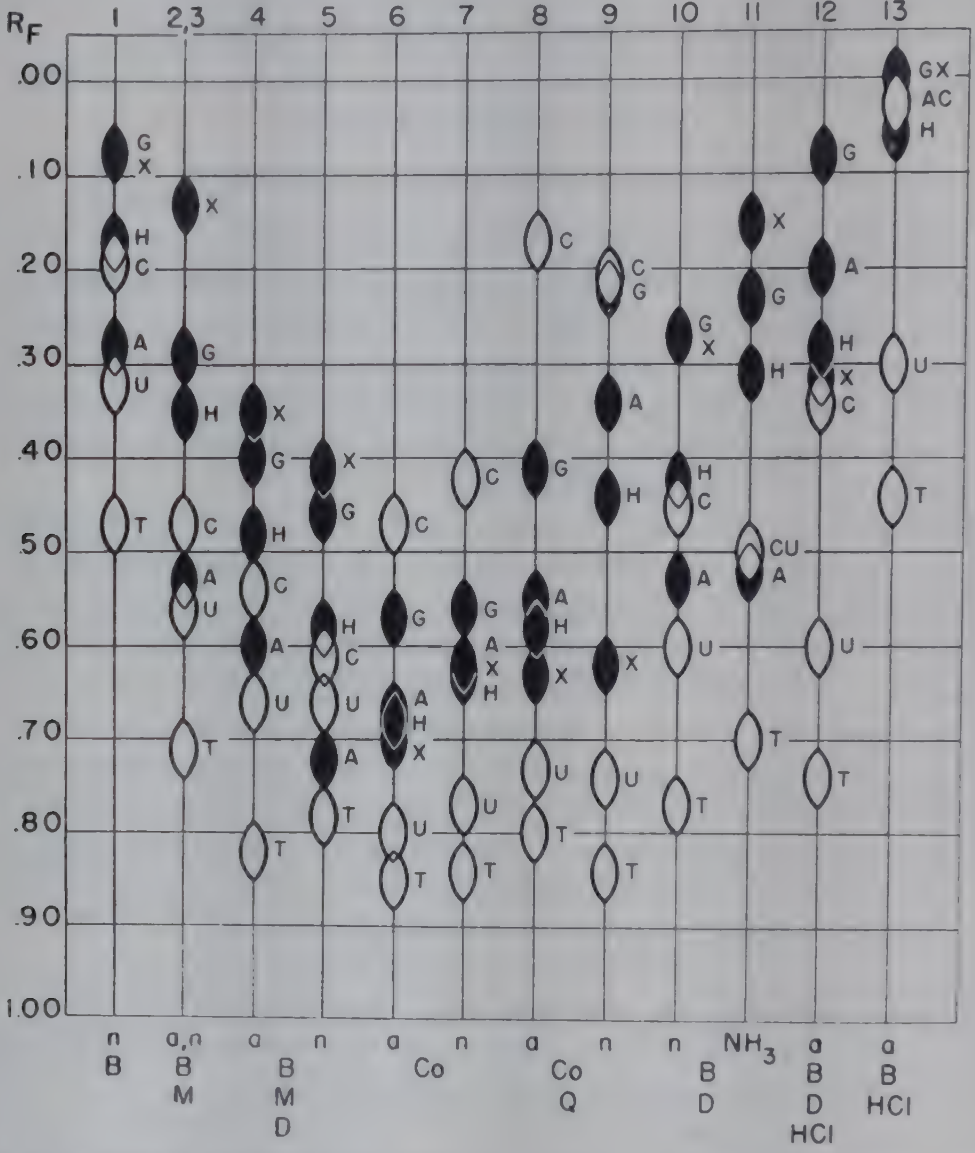


FIG. 1. Schematic representation of the position on the paper chromatogram of the purines and pyrimidines following the separation of a mixture. The numbering of the columns corresponds to the experiment numbers in Table I. *A* adenine, *G* guanine, *H* hypoxanthine, *X* xanthine, *U* uracil, *C* cytosine, *T* thymine. The conditions under which the separations were performed are indicated at the bottom. *a* acidic, *n* neutral, *B* *n*-butanol, *M* morpholine, *D* diethylene glycol, *Co* collidine, *Q* quinoline.

As presented here, the method applies to the separation and microestimation of all purines and pyrimidines normally found as constituents of nucleic acids, *i.e.* adenine, guanine, uracil, cytosine, and thymine. The separation and identification of xanthine and hypoxanthine likewise are included, but the description of the quantitative determination of these purines, which has been achieved by similar methods, will be presented later in another connection. The procedures can doubtless be extended to other substances with characteristic absorption, such as uric acid, the pterins, etc. The following paper (13) and investigations on various desoxypentose nucleic acids to be published shortly will illustrate the application of the method to the study of nucleic acid composition. Other uses, especially for the determination of individual purines and pyrimidines in tissues and body fluids, the separation of nucleotides, etc., will be discussed at subsequent occasions.

The procedures described here should, by offering a map, as it were, of the purine and pyrimidine composition of a mixture, be particularly adapted to the detection of unexpected components. The range within which the bases can be separated and determined lies between 5 γ and 40 to 50 γ. At the optimum concentration, *i.e.* with about 20 γ of each substance, the accuracy is ±4 per cent for the purines and even better for the pyrimidines, if the averages of a large series of determinations are considered. In individual estimations the accuracy is about ±6 per cent. Larger amounts cannot be separated satisfactorily, because then the adsorption zones tend to become diffuse. Should the necessity arise to demonstrate very small amounts of one purine or pyrimidine in the presence of relatively large quantities of the others, it would, therefore, be advantageous to resort to a preliminary fractionation of the bases by different means before the fractions are subjected to the separation procedures discussed here.

Attention may be directed to a comparison of the efficiency of different solvent systems in effecting separation, as illustrated in Fig. 1. This may be of interest, since the choice of solvents will necessarily be governed by the type of separation that is to be performed.

EXPERIMENTAL

Material

Adenine was a synthetic preparation (14), obtained through the courtesy of Dr. M. Hoffer of Hoffmann-La Roche, Inc., Nutley, New Jersey.

Guanine (Eastman Kodak Company) was three times recrystallized from HCl as the hydrochloride and then regenerated.

Hypoxanthine was a synthetic preparation.[1] *Xanthine* (Eastman Kodak Company) was twice recrystallized from water.

Uracil and *thymine* (Schwarz Laboratories, Inc., New York) were twice recrystallized from water.

---

[1] We are grateful to Dr. J. A. Aeschlimann, Hoffmann-La Roche, Inc., Nutley, New Jersey, for this specimen.

Cytosine was prepared from uracil by the method of Hilbert and Johnson (15).[2] It was twice recrystallized from water.

All substances used gave satisfactory elementary analyses.

The solvents employed were commercial preparations. *Morpholine* (Carbide and Carbon Chemicals Corporation) was rectified, b.p. 138°. *Quinoline* and *collidine* (crude), both supplied by the Koppers Company, Inc., were distilled.

The *filter paper* used for chromatography was Schleicher and Schüll, No. 597.

Selection of Solvent Systems for Separation

In Table I, the positions on the paper chromatogram of the several purines and pyrimidines, examined in a variety of solvent mixtures, are indicated. This is done by listing the R_F values (12), *i.e.* the proportion of the distances of the starting point from the adsorbate and from the solvent front. These values were determined at room temperature (*i.e.* at about 22°) with solutions containing only one component and were verified with mixtures.

The choice of solvent will, of course, vary with the particular problem, as can be gathered from Fig. 1, which illustrates graphically the positions of the four purines and three pyrimidines following the separation of the mixture in various solvents. Adenine and guanine may be separated from each other in all solvent systems examined, with the exception of collidine (Experiments 6 and 7) and collidine-quinoline (Experiments 8 and 9). Xanthine, on the other hand, is best demonstrated in neutral solution in collidine-quinoline (Experiment 9). For hypoxanthine collidine-quinoline (in neutral solution), butanol, or butanol-diethylene glycol can be used. The separation, for qualitative purposes, of the four purines may be performed under the conditions expressed in Experiments 5 and 11 of Table I.

Almost all solvents examined may serve for the separation of the pyrimidines from each other. In the presence of purines, cytosine can be demonstrated in collidine or collidine-quinoline, uracil and thymine in butanol-HCl, both with and without the admixture of diethylene glycol. It may be mentioned that isocytosine was found to have an R_F value in butanol very similar to that of uracil (Table I, Experiment 1).

Separation and Quantitative Estimation of Adenine and Guanine

Solutions—Because of the scanty solubility of guanine at neutrality, solutions of pH 0.8 to 1.0, usually in 0.1 N sulfuric acid, were used for the separation. Their concentration was 0.1 to 0.3 per cent with respect to each component. As a rule, 10 to 30 γ of each purine were contained in 0.01 to 0.02 cc., which was the volume serving for the individual separations. For comparative purposes, it was found important to employ similar volumes of solutions of the same degree of acidity.

---

[2] We are indebted to Dr. F. Misani for the synthesis of this substance.

TABLE I

Separation of Purines and Pyrimidines in Different Solvent Systems

Experiment No.	Mixture for separation*	Solvent system†	R_F values						
			Adenine	Guanine	Hypoxanthine	Xanthine	Uracil	Cytosine	Thymine
1	*n*	*n*-Butanol (saturated with water)	0.28	0.074	0.17	0.071	0.32	0.19	0.47
2	*a*	*n*-Butanol (3), morpholine (1), water (4)	0.53	0.29	0.35	0.13	0.56	0.47	0.71
3	*n*		0.53	0.29	0.35	0.14	0.55	0.46	0.73
4	*a*	*n*-Butanol (4.5), morpholine (1.5), diethylene glycol (1), water (2)	0.60	0.40	0.48	0.35	0.66	0.54	0.82
5	*n*		0.72	0.46	0.58	0.41	0.66	0.61	0.78
6	*a*	Collidine (saturated with water)	0.67	0.57	0.68	0.70	0.80	0.47	0.85
7	*n*		0.62	0.56	0.63	0.63	0.77	0.42	0.84
8	*a*	Collidine (1), quinoline (2) (mixture saturated with 1.5 parts water)	0.55	0.41	0.58	0.63	0.73	0.17	0.80
9	*n*		0.34	0.22	0.44	0.62	0.74	0.21	0.84
10	*n*	*n*-Butanol (4), diethylene glycol (1), water (1)	0.53	0.27	0.42	0.27	0.60	0.45	0.77
11	*n*		0.52	0.23	0.31	0.15	0.50	0.50	0.70
12	*a*	*n*-Butanol (4), diethylene glycol (1), 0.1 N HCl (1)	0.20	0.08	0.29	0.31	0.60	0.34	0.74
13	*a*	*n*-Butanol (saturated with 0.1 N HCl)	0.032	0.006	0.054	0.09	0.30	0.035	0.44

* *a* = acidic (test mixture of purines and pyrimidines dissolved in 0.1 N H_2SO_4); *n* = neutral (mixture neutralized on paper with gaseous NH_3 before chromatography). In Experiment 11 the separation was carried out in an NH_3 atmosphere.

† The figures in parentheses indicate volume proportions.

Separation—Paper sheets, 15 cm. wide and 50 cm. long, were divided, by ruling, into five 2.7 cm. wide longitudinal lanes. A transverse line, about 8 cm. below the top of the sheet, indicated the starting points at which, in the centers of four of the lanes, known volumes of the solution were deposited. The solutions were dispensed by means of a micro burette allowing the measurement of 0.01 cc. with an accuracy of ±1 per cent.

A micrometric burette of the Scholander type (16) or a "Gilmont ultramicroburet" (Emil Greiner Company, New York) was used. The fifth lane was left free.

Two solvent mixtures were employed in the quantitative determinations. One consisted of 4.5 parts (by volume) of *n*-butanol, 2 parts of water, 1.5 part of morpholine, and 1 part of diethylene glycol. The other mixture contained 4 parts of *n*-butanol and 1 part each of diethylene glycol and of water. When the latter solvent system was employed, the acidic purine solution was, after deposition on the paper, neutralized with gaseous ammonia[3] and the separation carried out in an ammonia atmosphere. The R_F values found with these solvent systems are included in Table I as Experiments 4 and 11. The experiments were carried out at room temperature in closed cylindrical Pyrex glass jars, 46 cm. high with an inside diameter of 21 cm. Two paper sheets were used simultaneously, their upper rims dipping, by means of suitable supports, into a trough filled with the solvent mixture. A beaker containing the same solvent was placed at the bottom of the jar. A second vessel contained N ammonia, if a NH_3 atmosphere was to be employed. The separation was terminated when the solvent front had almost reached the lower rim of the sheets, which ordinarily required about 20 hours.

Development—The paper sheets were first dried in air. The center column was then cut out, dried in an oven at 105° for 20 minutes, and sprayed with a 0.25 M solution of mercuric nitrate in 0.5 N nitric acid. The purines, thus fixed on the paper as Hg complexes, were made visible in the following manner. The sprayed paper strip was placed in a bath of 0.5 N nitric acid through which a slow stream of water was permitted to flow. The washing was considered as terminated when small paper strips, which, serving as controls, had also been sprayed with $Hg(NO_3)_2$ and put into the same bath, failed to blacken on treatment with ammonium sulfide. The chromatography strip was then passed through a solution of ammonium sulfide. Well defined black spots of mercuric sulfide indicated the position of the separated purines. As little as 5 γ could be demonstrated in this manner.

Extraction[4]—With butanol-morpholine-diethylene glycol-water as the solvent, the four remaining untreated lanes were cut apart, kept for 15 minutes in an atmosphere of ammonia, in order to neutralize traces of acid, and dried in an oven at 105° until no more visible vapors were given off. With the developed center strip serving as a guide, small rectangles (usually 5 to 6 cm. long) were removed from the untreated strips at the positions of purine adsorption. Each rectangle was placed in a small test-tube (13 × 100 mm.) and kept anew in an NH_3 atmosphere for 15 minutes. The tubes were put in a water bath, maintained at 80°, and 1 cc. of absolute ether was introduced, in three portions, into each tube, in order to remove by volatilization the last traces of morpholine. After the ether had evaporated completely, exactly 4 cc. of 0.1 N hydrochloric acid were added to each tube containing adenine and 4 cc. of N hydrochloric acid to the tubes containing guanine, and the closed vessels were kept overnight at 37°. The extracts then were well mixed, cooled to room temperature, and centrifuged immediately before spectroscopy.

---

[3] The purine samples that are neutralized on the paper before chromatographic separation must not contain more than 10 γ of guanine. Larger amounts of this difficultly soluble purine cannot, under neutral conditions, be recovered quantitatively, if contained in only 0.01 cc. of purine solution. For the recovery of larger amounts of guanine (compare Experiment 4 in Table III), it was found advisable to deposit two 0.01 cc. portions of solution side by side, in which case lanes 3.5 cm. wide were employed for the separation.

[4] The optical contamination of papers and extracts, for instance by contact with vapors of substances absorbing in the ultraviolet, must be avoided.

The use of butanol-diethylene glycol-water as the solvent rendered the extraction much simpler. The paper strips were dried in air for 3 to 4 hours and the paper segments, corresponding to the position of the separated purines, directly extracted, without further treatment, with 4 cc. portions of 0.1 N HCl (for adenine) or N HCl (for guanine).

Ultraviolet Spectroscopy—The absorption in the ultraviolet of the extracts was read, in 1 cm. quartz cells, in a Beckman photoelectric quartz spectrophotometer. The HCl extract of the corresponding paper rectangles removed from the fifth lane that had been left free, as explained above, served as the blank. Acidic extracts of filter paper themselves exhibit a low, but neither constant nor regular, absorption in the ultraviolet. For this reason, it was preferable, rather than taking the absolute extinction values at the absorption maxima (adenine at 262.5 mμ, guanine at 249 mμ) as the bases of calculation, to estimate the purine contents of the extracts from the difference in the extinction values read at the absorption maximum and at 290 mμ. For 0.001 per cent test solutions in 0.1 N HCl, *i.e.* for 10 γ of purine per cc., the difference Δ was determined as follows.

Adenine, $E_{262.5}$ 0.930; E_{290} 0.030; $\Delta = 0.900$
Guanine, E_{249} 0.737; E_{290} 0.262; $\Delta = 0.475$

In order to verify the position of the maximum, the ultraviolet absorption of the extracts was invariably also determined at 5 mμ above and below the characteristic absorption maximum of the purine in question, *i.e.* at 267.5 and 257.5 mμ for adenine, at 254 and 244 mμ for guanine. In addition, the extinction of the extracts also was measured at 300 mμ, at which wave-length the purines absorb very little light. The extinction values found at 300 mμ should, therefore, be very low, usually between −0.010 and +0.040. Readings outside this range were indicative of contamination, and such extracts were discarded.

The recovery of adenine with butanol-morpholine-diethylene glycol-water as the solvent system and the readings recorded in a series of such determinations are exemplified in Table II.

The results of a typical separation of adenine and guanine by means of butanol-diethylene glycol-water are presented here as an example. A mixture of 13.6 γ of adenine and 10.08 γ of guanine was subjected to separation. The following extinction values were recorded at different wave-lengths.

TABLE II

*Recovery of Adenine with Butanol-Morpholine-Diethylene Glycol-Water As Solvent**

Experiment No.	Adenine subjected to chromatography	Extinction at wave-length					Δ_x	$\frac{10\Delta_x}{\Delta}$	Adenine recovered	
		300 mμ	290 mμ	267.5 mμ	262.5 mμ	257.5 mμ				
	γ							γ	γ	*per cent*
1	19.64	0.023	0.039	0.429	0.458	0.439	0.419	4.66	18.6	95
2	19.47	0.008	0.024	0.418	0.448	0.428	0.424	4.71	18.8	97
3	19.96	0.007	0.023	0.412	0.442	0.428	0.419	4.66	18.6	93
4	19.47	0.013	0.026	0.408	0.428	0.411	0.402	4.47	17.9	92
5	19.64	0.019	0.036	0.434	0.460	0.440	0.424	4.71	18.8	96
6	19.64	0.016	0.031	0.429	0.455	0.438	0.424	4.71	18.8	96

* Δ_x is the difference in the extinction of the unknown at 262.5 and at 290 mμ, Δ the same difference for a standard solution containing 10 γ of adenine per cc. The expression $10\Delta_x/\Delta$ corresponds to the adenine concentration in 1 cc. of the unknown and, therefore, to one-fourth of the total recovered adenine.

Adenine		Guanine	
300 mμ	0.005	300 mμ	0.005
290 "	0.017	290 "	0.047
267.5 mμ	0.293	254 "	0.150
262.5 "	0.311	249 "	0.164
257.5 "	0.300	244 "	0.156

The computations which follow were based on the proportion between the Δ_x values found for the unknown and the Δ values determined, as explained above, with purine solutions containing 10 γ per cc.

Adenine, $\Delta_x = 0.311 - 0.017 = 0.294$; recovered in 1 cc. ($10\ \Delta_x/\Delta = 2.94/0.900$), 3.27 γ; total recovered in 4 cc., 13.1 γ; recovery, 96 per cent.

Guanine, $\Delta_x = 0.164 - 0.047 = 0.117$; recovered in 1 cc. ($10\ \Delta_x/\Delta = 1.17/0.475$), 2.46 γ; total recovered in 4 cc., 9.84 γ; recovery, 98 per cent.

The results of several similar separation experiments are listed in Table III.

Separation and Identification of Adenine, Guanine, Hypoxanthine, and Xanthine

It will suffice to mention only those points in which the procedures differed from the quantitative method described above. The solutions employed (in 0.1 N H_2SO_4) were 0.1 to 0.3 per cent with respect to each of the four purines. The solvent systems used for the separation are listed in Table I.

The development of the guide strips was in all experiments carried out as described above for the quantitative estimations, with the exception of Experiments 8 and 9 (Table I) in which the paper strips were briefly washed with ether before being sprayed with mercuric nitrate, since quinoline interfered with the development.

TABLE III

Separation of Mixtures of Adenine and Guanine by Means of n-Butanol-Diethylene Glycol-Water in NH_3 Atmosphere

Experiment No.	Adenine			Guanine		
	Amount in mixture	Amount recovered		Amount in mixture	Amount recovered	
	γ	γ	*per cent*	γ	γ	*per cent*
1	13.6	13.8	101	10.1	10.1	100
2	13.6	12.9	95	10.1	10.4	103
3	13.6	12.9	95	10.1	9.9	98
4	27.2	25.4	93	20.1	19.0	95

For the extraction of the adsorbates, the strips were, in Experiments 1 and 10 to 13 (Table I), dried in air for 4 hours, and then divided into segments and extracted as described above. In Experiments 2 to 5 the procedures employed for the quantitative estimation with butanol-morpholine-diethylene glycol as the solvent system were followed.

In Experiments 6 to 9 (Table I), *i.e.* with collidine or collidine-quinoline,[5] it was necessary to remove the last traces of these solvents which absorb strongly in the ultraviolet. This was done by steam distillation. The paper segment was placed in a test-tube and wetted with 2 N sodium carbonate, in order to liberate the solvent. Two 0.5 cc. portions of water were then permitted to evaporate from the tube in a bath of 110°. The subsequent extraction with 0.1 N hydrochloric acid was carried out in the same tubes.

---

[5] The use of a quartz lamp made it possible to ascertain the extent to which the strongly fluorescent quinoline sulfate had spread during the chromatography, since the separation was more complete when all purines had migrated below the acid zone.

In many of the separation experiments the complete absorption curves of the HCl extracts were determined, in order to check the purity of the extracted bases.

Separation and Quantitative Estimation of Uracil, Cytosine, and Thymine

Solutions—Aqueous solutions of the pyrimidines served for the separation experiments. The volumes deposited on the paper were 0.01 to 0.02 cc., containing 10 to 30 γ of each component.

Separation—*n*-Butanol, saturated with water, was employed for the separation, which required about 12 hours. Otherwise, the procedures were identical with those followed in the quantitative purine separation. The R_F values of the separated pyrimidines in several solvent mixtures are included in Table I.

Development—The paper sheet was spread out and dried in air and the center column cut out and dried in an oven at 105° for 20 minutes. It then was placed for about 30 seconds in a buffered 0.01 M mercuric acetate solution of pH 6.2. This solution was freshly prepared by mixing 1 part of 0.1 M mercuric acetate solution with 3 parts of M sodium acetate solution and 6 parts of water. The strip, after having been bathed for exactly 20 seconds in slowly renewed water, was passed through an ammonium sulfide solution. Compact spots of mercuric sulfide denoted the positions of the separated pyrimidines. The identification limits were about 5 γ for uracil and cytosine, 10 γ for thymine.

Extraction—The paper strips were dried in air for 4 hours and the 3.5 to 5 cm. long paper rectangles, cut out with the guidance of the developed strip, were each extracted with exactly 4 cc. of water in closed tubes that were kept overnight at 37°. The well mixed extracts were centrifuged before spectroscopy.

Ultraviolet Spectroscopy—The principles discussed above with respect to the estimation of the purines apply here too. The concentrations of cytosine and thymine were determined, as for the purines, from the difference in the extinction values found at the respective absorption maxima and at 290 mμ. For uracil the difference between the absorption maximum and the reading obtained at 280 mμ was used. The absorption maxima recorded for the preparations were uracil 259, cytosine 267.5, and thymine 264.5 mμ. The following values for the difference Δ were found with 0.001 per cent solutions of the pyrimidines in distilled water.

Uracil, E_{259} 0.738; E_{280} 0.148; $\Delta = 0.590$
Cytosine, $E_{267.5}$ 0.598; E_{290} 0.053; $\Delta = 0.545$
Thymine, $E_{264.5}$ 0.626; E_{290} 0.081; $\Delta = 0.545$

As was explained before with regard to the purines, additional absorption readings at 5 mμ above and below the respective maxima and also at 300 mμ served to ascertain the purity of the extracts. The readings recorded in a typical separation of uracil, cytosine, and thymine are exemplified below. Table IV summarizes the results of five separation experiments.

TABLE IV

Separation of Mixtures of Uracil, Cytosine, and Thymine

Experiment No.	Uracil			Cytosine			Thymine		
	Amount in mixture	Amount recovered		Amount in mixture	Amount recovered		Amount in mixture	Amount recovered	
	γ	γ	*per cent*	γ	γ	*per cent*	γ	γ	*per cent*
1	24.3	23.1	95	24.8	23.9	96	24.8	24.5	99
2	25.0	23.7	95	25.0	24.5	98	25.0	23.6	95
3	24.5	24.5	100	24.3	24.6	101	24.5	23.1	94
4	11.9	11.9	100	10.7	10.5	98	11.9	11.9	100
5	11.8	11.4	97	10.5	9.9	94	11.8	11.2	95

TABLE V

Extinction Values for Eluates of Separated Pyrimidines

Uracil		Cytosine		Thymine	
Wave-length	*E*	Wave-length	*E*	Wave-length	*E*
mμ		*mμ*		*mμ*	
300	0.004	300	0.003	300	−0.003
280	0.046	290	0.014	290	0.016
264	0.210	272.5	0.150	269.5	0.172
259	0.222	267.5	0.157	264.5	0.178
254	0.212	262.5	0.153	259.5	0.174

A mixture containing 11.9 γ of uracil, 10.7 γ of cytosine, and 11.9 γ of thymine was subjected to a chromatographic separation. The readings of the extinction values recorded for the eluates (4 cc.) of the separated pyrimidines are shown in Table V.

The computations, based on the proportion between the Δ_x values found for the unknown and the Δ values found for pyrimidine solutions containing 10 γ per cc., follow here.

Uracil, $\Delta_x = 0.222 - 0.046 = 0.176$; recovered in 1 cc. ($10\ \Delta_x/\Delta = 1.76/0.590$), 2.98 γ; total recovered in 4 cc., 11.9 γ; recovery, 100 per cent.

Cytosine, $\Delta_x = 0.157 - 0.014 = 0.143$; recovered in 1 cc. ($10\ \Delta_x/\Delta = 1.43/0.545$), 2.62 γ; total recovered in 4 cc., 10.5 γ; recovery, 98 per cent.

Thymine, $\Delta_x = 0.178 - 0.016 = 0.162$; recovered in 1 cc. ($10\ \Delta_x/\Delta = 1.62/0.545$), 2.97 γ; total recovered in 4 cc., 11.9 γ; recovery, 100 per cent.

The assistance of Miss Ruth Doniger and Mrs. Charlotte Green is gratefully acknowledged. In its first phases the work was supported by a grant from the Rockefeller Foundation.

SUMMARY

Mixtures containing minute amounts of purines (adenine, guanine, hypoxanthine, xanthine) and pyrimidines (uracil, cytosine, thymine) were separated in a variety of solvent systems. The method developed for this purpose, which makes use of paper chromatography, permits not only the demonstration of the individual components by their conversion into mercury salts, but also their identification and quantitative estimation by means of ultraviolet spectroscopy. Amounts ranging from 5 to 40 γ of adenine, guanine, uracil, cytosine, and thymine thus were separated and determined quantitatively.

BIBLIOGRAPHY

1. Vischer, E., and Chargaff, E., *J. Biol. Chem.*, **168,** 781 (1947).
2. Vischer, E., and Chargaff, E., *Federation Proc.*, **7,** 197 (1948).
3. Chargaff, E., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **12,** 28 (1947).
4. Jorpes, E., in Bamann, E., and Myrbäck, K., Die Methoden der Fermentforschung, Leipzig, **1,** 1090 (1941).
5. Salkowski, E., *Arch. ges. Physiol.*, **69,** 268 (1897–98).
6. Krüger, M., and Schmid, J., *Z. physiol. Chem.*, **45,** 1 (1905).
7. Graff, S., and Maculla, A., *J. Biol. Chem.*, **110,** 71 (1935).
8. Schmidt, G., *Z. physiol. Chem.*, **219,** 191 (1933).
9. Kalckar, H. M., *J. Biol. Chem.*, **167,** 429, 445 (1947).
10. Loring, H. S., Ordway, G. L., Roll, P. M., and Pierce, J. G., *Federation Proc.*, **6,** 510 (1947).
11. Loring, H. S., and Fairley, J. L., Jr., *J. Biol. Chem.*, **172,** 843 (1948).
12. Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, **38,** 224 (1944).
13. Vischer, E., and Chargaff, E., *J. Biol. Chem.*, **176,** 715 (1948).
14. Hoffer, M., Jubilee volume Emil Barell, Basle, 428 (1946); *Chem. Abstr.*, **41,** 4108 (1947).
15. Hilbert, G. E., and Johnson, T. B., *J. Am. Chem. Soc.*, **52,** 1152 (1930).
16. Scholander, P. F., *Science*, **95,** 177 (1942).

THE COMPOSITION OF THE PENTOSE NUCLEIC ACIDS OF YEAST AND PANCREAS*

By ERNST VISCHER† and ERWIN CHARGAFF

(*From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York*)

(Received for publication, June 30, 1948)

The historically understandable attempts to simplify the problem of nucleic acid structure by the formulation of the tetranucleotide hypothesis have found their classical exposition in Levene's monograph of 1931 (1). The same tendencies are expressed, less precisely, in statements found in many text-books according to which the nucleic acid unit, having a molecular weight of about 1200, contains four different bases (two purines and two pyrimidines) in equimolecular proportions. With the growing recognition of the complex and macromolecular character of the nucleic acids the necessity for the postulation of these hypothetical units vanished and it became possible to consider nucleic acids as substances comparable to the proteins in intricacy and, perhaps, even in specificity.

Our present knowledge of the structure of nucleic acids has been reviewed repeatedly in the recent past (2–4). Other relevant aspects also have been considered (5, 6).

The method for the quantitative estimation of individual purines and pyrimidines in mixtures of these bases, presented in the preceding paper (7), has made possible a new approach to the study of the composition of nucleic acids. The present communication describes the application of these procedures to a study of the distribution of the nitrogenous constituents of the pentose nucleic acids of yeast and pig pancreas and includes a consideration of aspects related to this problem, such as the mechanisms of the acid hydrolysis of these compounds. Other accounts, to be submitted shortly, will deal with the composition of desoxypentose nucleic acids. The results reported here will, later in this paper, be correlated with the findings of previous workers.

EXPERIMENTAL

Material

Ribonucleic Acid of Yeast—The purification procedure used was a modification of that of Fletcher *et al.* (8). 15 gm. of yeast nucleic acid (Merck)

---

* This work has been supported by a research grant from the United States Public Health Service.

† Swiss-American Student Exchange Fellow.

were dissolved in 400 cc. of water by the addition of 15 cc. of 2 N ammonia. The filtered solution (pH 6.2) was dropped, with mechanical stirring, into 860 cc. of 95 per cent ethanol containing 5 cc. of concentrated HCl. The precipitate, collected by centrifugation, was washed with 125 cc. of 66 per cent ethanol and redissolved in 250 cc. of water and 16 cc. of 2 N ammonia. The precipitate obtained from the filtered solution with 500 cc. of alcohol and 4 cc. of concentrated HCl was suspended in 25 cc. of water and subjected to dialysis against running water (14 hours) and several changes of ice-cold distilled water (20 hours). The ribonucleic acid was collected, washed with 66, 98, and 100 per cent ethanol and with ether, and dried *in vacuo* over P_2O_5; it weighed 7.2 gm. For analysis, the substance was dried in a high vacuum at 60° for 3 hours and weighed out under exclusion of moisture.

Found, N (Dumas) 15.3, P (Pregl-Lieb) 8.0

This nucleic acid showed the characteristic absorption spectrum in the ultraviolet (pH 6.5). For the maximum at 257.5 mμ an ϵ(P) value of 9800 was found; at the minimum of 229 mμ ϵ(P) was 4200 (for definitions, see (9)).

Pentose Nucleic Acid of Pig Pancreas[1]—The preparation of this material followed in all essentials the procedures described by Jorpes (10, 11) and by Levene and Jorpes (12). The free pentose nucleic acid gave no protein tests and contained only a small amount (3 per cent) of desoxypentose nucleic acid (diphenylamine reaction). The substance was, for analysis, dried as described above.

Found, N (Dumas) 15.4, P (Pregl-Lieb) 7.9

The absorption maximum of this preparation (pH 6.3) was at 256 mμ with an ϵ(P) of 9800; the corresponding value for the minimum at 228 mμ was 4200.

Ribose Nucleotides—Adenylic acid was prepared from commercial yeast nucleic acid (Schwarz Laboratories, Inc., New York) in the crystalline state by the recent method of Buell (13). A few modifications were introduced, which included the use of 0.5 N alkali for 24 hours at 37° for the cleavage of the nucleic acid, and the repeated precipitation of the nucleotide as the lead salt before the recrystallization of the free adenylic acid from water. The air-dried white crystalline substance contained N (Dumas) 18.54, P (Pregl-Lieb) 8.45 per cent. It lost 2.6 per cent of its weight when dried for 2 hours at 109° *in vacuo*.

Basic sodium guanylate was prepared as a white crystalline powder from the potassium acetate complex of guanylic acid, collected in the course of the preparation of adenylic acid, by the method of Steudel and Peiser (14). The material, dried at 77° *in vacuo*, contained N (Dumas) 13.27, P (Pregl-Lieb) 6.16 per cent.

---

[1] We are indebted to Dr. F. Misani for help with this preparation.

Cytidylic acid (Preparation 1) was prepared from the acid hydrolysate of commercial yeast nucleic acid by the fractional crystallization of the brucine salts according to Levene (15, 16). The nucleotide, recrystallized from 35 per cent ethanol and dried *in vacuo* at 65°, contained N 12.3, P 9.0 per cent, and had a rotation of $[\alpha]_D^{27} = +50.3°$ (0.1 per cent solution in water). Another specimen of cytidylic acid (Preparation 2) was obtained through the courtesy of Dr. S. J. Thannhauser and Dr. G. Schmidt. It contained, after recrystallization from 35 per cent ethanol, N 12.4, P 9.5 per cent, and had $[\alpha]_D^{27} = +50.8°$ (0.1 per cent solution in water). Levene (16) found for this nucleotide a rotation of $[\alpha]_D^{50} = +48.5°$.

Quantitative Estimation of Purines in Nucleic Acids

The nucleic acid was dried in a high vacuum at 60° for 3 hours and 5 to 8 mg. of the preparation, weighed on a micro balance, were placed in a small Pyrex bomb tube (160 × 5 mm.); 0.5 cc. of N sulfuric acid was added and the sealed tube heated for 1 hour at 100° in a boiling water bath. The clear solution was allowed to cool and was transferred, by means of a long capillary pipette, into a 1 cc. volumetric flask. The tube walls were rinsed three or more times with a few drops of 0.1 N H_2SO_4. The washings served to bring the hydrolysate up to volume. When the solvent system used for the subsequent purine separation consisted of *n*-butanol, morpholine, diethylene glycol, and water (7), the undiluted hydrolysate in the volumetric flask was first adjusted to pH 0.8 to 1 by means of a few drops of 30 per cent aqueous NaOH and then brought to a volume of exactly 1 cc. with 0.1 N H_2SO_4, the washings being used for this purpose. The pH adjustment was controlled by dipping the tip of a sliver of hydrion paper (pH 0 to 1.5) into the solution. When morpholine was omitted from the solvent mixture (7), no alkali was added and the solution was brought up to volume directly with 0.1 N H_2SO_4.

Several 0.01 to 0.02 cc. portions of this solution, dispensed on paper, as described in the preceding publication (7), by means of an accurate micrometric burette, served for parallel separations and estimations. As a rule, six determinations were carried out simultaneously with the same hydrolysis fluid. The procedures used for the development of the guide strip, which indicated the number and positions of the separated purines, and for the extraction and the identification and estimation of the components by spectroscopy in the ultraviolet followed exactly the methods described before (7).[2] In all analyses, a drop of the hydrolysate and a drop of a purine test mixture of known composition were chromatographed side by side on a separate paper sheet, in order to compare the positions of the adsorbates on the chromatogram and thereby to verify the identification of the separated components.

Quantitative Estimation of Pyrimidines in Nucleic Acids

A portion, weighing between 15 and 25 mg., of the nucleic acid, that had been dried in a high vacuum at 60° for 3 hours, was weighed exactly into the special vessel shown as *C* in Fig. 1. Absolute methanol (0.5 to 1 cc.) was added and dry HCl gas passed, by means of a capillary, through the suspension with complete exclusion of moisture. A copper spiral through which cold water circulated converted the neck of the flask into a reflux condenser. (The experimental arrangement is illustrated in Fig. 1.) Within about 30 minutes the nucleic acid dissolved in the methyl alcohol, which became warm, and the purine hydrochlorides began to precipitate. The passage of HCl gas then was continued for 3 to 5 hours while the mixture was kept at 50°. After being chilled overnight with rigorous exclusion of moisture, the closed reaction vessel was centrifuged. The supernatant was quantitatively transferred to a bomb tube (220 mm. long, inside diameter 65 mm., outside diameter 85 mm.) by means of a capillary siphon which was actuated by suction.[3]

The yellowish methanol solution was evaporated at about 45° under a nitrogen current and the evaporation was continued, with the addition of small portions of fresh methanol, until the alcohol vapors carried almost no acid. About fifteen evaporations were, as a rule, necessary. The residue was dried overnight *in vacuo* over $CaCl_2$ and KOH. After the introduction of 0.5 cc. of concentrated formic acid (98 to 100 per cent, Eastman Kodak), the bomb tube was sealed and heated at 175° for 2 hours. It then was chilled to 0° and, because of inside pressure, opened with all necessary precautions.

---

[2] It might be pointed out that it is possible to ascertain the presence on the chromatogram of minor purine or pyrimidine components whose concentrations are insufficient to permit their direct demonstration as Hg salts. As the relative positions of the individual purines and pyrimidines on the chromatogram are known (7), the extract of a segment, removed from the paper strip at the location of the suspected substance, may serve for the spectroscopic examination. In this manner, the pentose nucleic acid of pancreas was tested for thymine, but none was found.

[3] It was found preferable to omit the washing of the purine hydrochloride sediment, since this resulted invariably in the contamination of the pyrimidine fraction by purines, even when cold methanol saturated with gaseous HCl served as the wash fluid. The precipitated purine hydrochlorides, dissolved in 0.1 N H_2SO_4 and brought up to a known volume, may be subjected to separation by chromatography and estimation. The results, however, were not as constant as when the purine hydrolysis was carried out with N H_2SO_4, as described in the preceding section.

To the dark brown hydrolysate 2 to 3 drops of about 40 per cent NaOH solution were added, which effected the flocculation of the pigment and the clarification of the solution.[4] The tube was centrifuged, the light yellow supernatant transferred to a 1 cc. volumetric flask, and the centrifugation residue washed, at least three times, with 0.1 to 0.2 cc. portions of warm water. The centrifuged washings were used to bring the volume of the hydrolysate up to 1 cc. Several, usually six, exactly measured 0.01 to 0.02 cc. portions of this solution were deposited on the paper sheets serving for the chromatographic separation and were neutralized with gaseous ammonia. The separation with *n*-butanol-water as the solvent, the development, the extraction, and the identification and quantitative determination

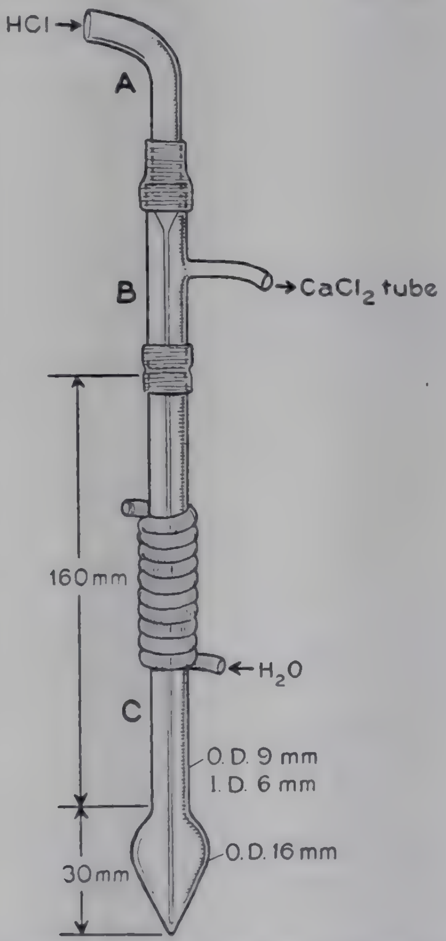


Fig. 1. Arrangement for the hydrolysis of very small amounts of nucleic acid The capillary tube *A* is connected through the adapter *B* with the reaction vessel *C*.

---

[4] When insufficiently clarified brown solutions are subjected to chromatography, dark tongues form on the paper which interfere with the quantitative spectroscopy.

of the separated pyrimidines were carried out by the method previously described (7).

Control Experiments

Resistance of Pyrimidines to Acid Treatment—It is well known that the liberation of pyrimidines from nucleic acids requires an extremely drastic treatment, *e.g.* autoclaving at 175° for several hours with 20 per cent HCl (17) or 25 per cent H_2SO_4 (18). It appeared of interest to take advantage of the ease with which changes in the composition of pyrimidine mixtures can be followed with the new chromatographic method (7) by studying the effect of various acids on a mixture of uracil, cytosine, and thymine.

TABLE I

Resistance of Pyrimidines to Treatment with Strong Acid

A mixture of pyrimidines of known concentration was dissolved in the acids indicated below and heated at 175° in a bomb tube. The concentration shifts of the individual pyrimidines were determined through a comparison of the recoveries of separated pyrimidines before and after the heating of the mixture.

Experiment No.	Acid	Heating time	Concentration shift, per cent of starting concentration		
			Uracil	Cytosine	Thymine
		min.			
1	HCl (10%)	90	+62	−63	+3
2	10 N HCOOH + N HCl (1:1)	60	+3	−5	0
3		120	+24	−19	0
4	HCOOH (98 to 100 %)	60	0	−1	−2
5		120	0	+2	+1

The acids examined were 10 per cent hydrochloric acid, a mixture of equal parts of 10 N formic and N hydrochloric acids (19), and, finally, pure formic acid (98 to 100 per cent).

The original solution that served for the experiments contained 49.1 mg. of uracil, 50.4 mg. of cytosine, and 44.4 mg. of thymine in 10 cc. of 10 per cent HCl. A 2 cc. aliquot of this solution was neutralized with concentrated aqueous NaOH and diluted with water to an exact volume of 5 cc. Several 0.01 cc. portions of this neutral solution were subjected to chromatographic separation on filter paper with *n*-butanol-water as the solvent and to quantitative spectroscopic examination of the separated components (7). Another 2 cc. aliquot of the original test mixture was heated in a bomb tube for 90 minutes at 175°, neutralized and diluted to 5 cc., as described before, and likewise subjected to quantitative separation. The experiments with other acids were carried out in a similar manner. The results, assembled in Table I, indicate the instability of cytosine, which was to a large extent converted to uracil, in acids other than formic acid; uracil and thymine resisted the acid treatment.

Hydrolysis of Yeast Ribonucleic Acid with Strong HCl—Following the liberation of purines by hydrolysis with gaseous HCl in dry methanol and the concentration of the supernatant from the purine hydrochlorides to dryness, as described above, the pyrimidine nucleotide residue was heated, in a sealed tube, with 0.3 cc. of 10 per cent hydrochloric acid at 175° for 2 hours. The pyrimidines were separated in the usual manner. Under these conditions the nucleic acid yielded 2.1 per cent of cytosine, 7.0 per cent of uracil. A comparison with the quantitative estimations summarized in Table VI will show that the hydrolysis with strong mineral acid produced an enormous shift in the relative proportions of the two pyrimidines, although the total amount recovered was nearly the same. In the HCl hydrolysate the mole proportions of pyrimidine to phosphoric acid were cytosine 0.073, uracil 0.242; the cytosine N amounted to only 5.2, the uracil N to 11.4 per cent of the nucleic acid N; the molar cytosine to uracil ratio was 0.3.

Hydrolysis of Yeast Ribonucleic Acid with Formic and Hydrochloric Acids—The following experiments were designed to exclude the possibility that the attacks on the nucleic acid by formic and by hydrochloric acids were directed against different groupings and were selective with respect to the proportions of liberated pyrimidines. From 48.052 mg. of yeast ribonucleic acid (dried in a high vacuum) the purines were split off as the hydrochlorides, in the manner described, by means of methanolic HCl. The methanolic supernatant was evaporated and freed of HCl and the residue transferred to a 2.5 cc. volumetric flask and dissolved in methanol. Two 1 cc. portions of this solution, designated Solutions A and B, were subjected to hydrolysis. Solution A was taken to dryness, the residue heated with 1 cc. of concentrated formic acid for 2 hours at 175° in a sealed bomb tube, and the pyrimidine distribution determined in the usual manner. The evaporation residue of Solution B was similarly treated with formic acid. The hydrolysate was evaporated (at the end with frequent additions of absolute ethanol) under nitrogen at 45° and the residue again subjected to hydrolysis in a sealed tube, this time with 1 cc. of 20 per cent HCl, at 175° for 2 hours. The following results on the pyrimidine distribution again confirmed the destructive effect of mineral acids on cytosine, but ruled out the assumption of a differential action of formic and hydrochloric acids: Solution A, cytosine 6.8, uracil 2.4, total pyrimidines 9.2 per cent; Solution B, 3.5, 5.7, and 9.2 per cent, respectively.

Hydrolysis of Nucleotides—3.264 mg. of *adenylic acid* were hydrolyzed with N sulfuric acid at 100° for 1 hour as described above. Aliquots corresponding to 32.64 γ of the nucleotide yielded, when chromatographed in *n*-butanol-diethylene glycol-water, 11.1 γ of adenine (95 per cent of the amount expected from the N content). *Sodium guanylate*, similarly treated, gave, per 29.08 γ of the nucleotide, 8.34 γ of guanine (100 per cent).

The hydrolysis of *cytidylic acid* was studied with the two preparations mentioned before. With Preparation 1 the liberation of phosphoric acid and of cytosine by treatment with concentrated formic acid for various periods was followed and compared with the behavior of yeast ribonucleic acid under analogous conditions. Both the nucleotide and the nucleic acid were subjected to the operations necessary for the removal of the purines, the hydrolysis with formic acid at 175° in a bomb tube, and the determination of the pyrimidines in the manner described. At the same time, the total and the inorganic phosphorus contents were estimated colorimetrically in dilutions of the hydrolysates. The results will be found in Table II. Another set of experiments with cytidylic acid (Preparation 2), reproduced in Table III, was designed to compare the effects of 90 and 99 per cent formic acids and of very strong hydrochloric acid. All hydrolyses were performed in the customary manner in bomb tubes at 175°. It will be seen (Experiment 1a) that the action of fresh acid on a hydrolysate produced little additional effect. The behavior of cytidylic acid preparations toward hydrolysis obviously will require additional study. A glance at Tables II and III will show that the recovery of cytosine amounted in no case to more than about 80 per cent of the nucleotide nitrogen. It was even lower with strong hydrochloric acid as the hydrolyzing agent when, as expected, a portion of the cytosine was converted to uracil (Experiment

Table II

Liberation of Pyrimidines and of Phosphorus from Yeast Ribonucleic Acid and from Cytidylic Acid by Heating with Concentrated Formic Acid

Duration of heating at 175°	Yeast ribonucleic acid		Cytidylic acid		
	Inorganic P	Total pyrimidines (cytosine + uracil)	Inorganic P	Cytosine	Cytosine N
min.	*per cent of total P*	*per cent*	*per cent of total P*	*per cent*	*per cent of nucleotide N*
0	6		0		
30	83				
60	85		76		
90	100	8.2	100	22.7	70
120	100	9.2	100	25.8	80
150				25.6	79
210		9.1			
300		9.2			

3 in Table III). The reasons for this refractoriness of cytidylic acid are not yet clear.

Composition of Yeast Ribonucleic Acid

Purines—The only purines encountered on the chromatograms were adenine and guanine. The absorption maximum of adenine (in 0.1 N HCl) was at 263 mμ, that of guanine (in N HCl) at 249 mμ. A series of estimations, each representing the average of at least six parallel determinations, is assembled in Table IV. The average of all adenine values reported is 9.1 per cent, that of all guanine values 10.2 per cent. If the value for guanine found for Hydrolysate 13 (Table IV), which appears rather out of range, is disregarded, the guanine average becomes 10.0 per cent. The experiment with Hydrolysate 22 is for several reasons considered as the most reliable; it will be seen that the figures reported for it differ only very slightly from the averages computed for the entire series. When adenine in Hydrolysate 4 and guanine in Hydrolysate 22 each were determined with the use of two different solvent systems, the results were in very good agreement.

TABLE III

Hydrolysis of Cytidylic Acid

Experiment No.	Acid	Duration of heating at 175°	Pyrimidines found			
			Cytosine	Cytosine N	Uracil	Uracil N
		hrs.	*per cent*	*per cent of nucleotide N*	*per cent*	*per cent of nucleotide N*
1	99% HCOOH	2	23.8	73	0	0
1a*	99% "	4	25.4	78	0	0
2	90% "	2	26.0	79	0	0
3	20% HCl	2	16.6	51	6.5	13

* An aliquot of the hydrolysate obtained in Experiment 1 was evaporated to dryness under N_2 at 45° and the residue heated with a fresh portion of concentrated formic acid for 2 hours. Only traces of absorbing material were found at the chromatographic position of uracil.

Pyrimidines—Cytosine and uracil, the latter a relatively minor component, were the pyrimidines found in the hydrolysates. The absorption maximum for cytosine was at 268 mμ, that for uracil at 259 mμ (solutions in water). The results of several determinations are summarized in Table V. The average value for cytosine was 6.7, that for uracil 2.3 per cent. These figures, it is felt, require an upward correction of 5 per cent. As was pointed out above (see also foot-note 3), it was found advantageous to omit the washing of the purine hydrochlorides that precipitated in the course of the hydrolysis with methanolic HCl, in order to avoid the contamination of the pyrimidine fraction. This involved, of course, a small loss in pyrimidines owing to the retention of some pyrimidine nucleotide by the purine sediment. For an estimate of the extent of this loss, hydrolysates of thymus nucleic acid were employed, since the position of thymine on the chromatogram rendered it the pyrimidine least affected by contamination with purines (7). It was thus found, in comparative hydrolysis experiments, that approximately 5 per cent of the thymine was retained in the purine hydrochloride precipitate. For this reason, the pyrimidine figures given in Tables VI and VIII, which summarize the distribution of the nitrogenous components of pentose nucleic acids, were corrected by this factor.

TABLE IV

*Purine Content of Yeast Ribonucleic Acid**

Hydrolysate No.	Solvent system	Adenine	Guanine
		per cent	*per cent*
1	*n*-Butanol-morpholine†	9.7	
2	"	8.6	
4	"	9.5	
4	*n*-Butanol-diethylene glycol-morpholine	9.5	
7	" "	9.2	
10	" "	9.9	
13	" "	9.6	12.2
14	" "	8.6	9.7
21	" "	8.1	9.3
22	" "	9.0	10.1, 10.4‡
24	" "		10.3
25	" "		10.0
27	" "	8.4	9.9

* Each value represents the average of at least six parallel determinations (concordant within a range of 5 per cent) on the same hydrolysate.

† This solvent system, described in the preceding paper (7) for qualitative separations, may be used for the estimation of adenine but not of guanine. The procedures are the same as for the solvent containing diethylene glycol.

‡ In this determination *n*-butanol-diethylene glycol (in NH_3 atmosphere) was employed as the solvent.

Proportions and Balances—Table VI provides a survey of the distribution of purines and pyrimidines in the yeast ribonucleic acid. The fact that the purines or pyrimidines liberated by the hydrolysis of a nucleotide contain 1 hydrogen atom more than the corresponding radicals present in the uncleaved compound was left out of consideration, since the contributions from this correction would have been negligible. It will be seen that the molar proportions (taking uracil as 1) and the mole per mole of phosphorus ratios of the nitrogenous constituents rule out the existence of a regular tetranucleotide. It is of course understood that the computations presented here are not at all dependent upon any particular assumption regarding the structure of the nucleic acid analyzed. That the recoveries in terms of total nitrogen and of total phosphorus were closely similar, but did not quite reach 100 per cent, may be significant, as will be pointed out later.

TABLE V

*Pyrimidine Content of Yeast Ribonucleic Acid**

Hydrolysate No.	Cytosine	Uracil
	per cent	*per cent*
32	†	2.1
34	7.4	2.3
35	6.5	2.4
36	6.1	2.3
38	6.8	2.4

* Each value represents the average of at least six parallel determinations (concordant within 5 per cent) on the same hydrolysate. In all separations *n*-butanol (saturated with water) served as the solvent.

† The absorption spectrum of the cytosine solution, isolated in this experiment, indicated contamination.

TABLE VI

*Yeast Ribonucleic Acid; Proportions and Balances**

Compound	Content in nucleic acid	Nitrogen in nucleic acid	N accounted for	$\frac{\text{Purine N}}{\text{Pyrimidine N}}$	Mole per mole P	P accounted for	Moles per 4 moles P	Molar proportions
	per cent	*per cent*	*per cent of nucleic acid N*			*per cent of nucleic acid P*		
Adenine	9.1	4.72	30.9		0.261	26.1	1.04	3.2
Guanine	10.0	4.63	30.3		0.256	25.6	1.02	3.1
Cytosine	7.0	2.65	17.3		0.244	24.4	0.98	3.0
Uracil	2.4	0.60	3.9		0.083	8.3	0.33	1.0
Total nucleic acid			82.4	2.9		84.4		

* The nucleic acid preparation contained N 15.3, P 8.0 per cent.

Composition of Pancreas Pentose Nucleic Acid

As was the case with the yeast nucleic acid discussed immediately above, adenine, guanine, cytosine, and uracil were the four nitrogenous constituents encountered on the chromatograms. Tests carried out with extracts collected in the region of thymine adsorption (compare foot-note 2) failed to indicate the presence of this pyrimidine. The series of estimations reproduced in Table VII revealed an average content of adenine 5.7, guanine 15.5, cytosine 5.5, uracil 1.2 per cent. Guanine, therefore, was by far the major component of this nucleic acid. The molar guanine to adenine ratio was 2.4. It might be mentioned that in the analysis of a second less pure preparation of a pancreas nucleic acid which is not discussed in detail here, since it contained some protein and about 6 per cent of desoxypentose nucleic acid, the following figures were found: adenine 4.1, guanine 16.5 per cent.

The proportions and balances, computed as for yeast ribonucleic acid, are tabulated in Table VIII.

TABLE VII

*Purine and Pyrimidine Content of Pancreas Pentose Nucleic Acid**

Hydrolysate No.	Adenine	Guanine	Cytosine	Uracil
	per cent	*per cent*	*per cent*	*per cent*
1	6.0	15.4		
2†	5.5	16.0		
3	5.6	15.2		
5			5.3	1.5
6			5.9	1.1
7			5.2	1.0

* Each value represents the average of at least six parallel determinations (concordant within 5 per cent) on the same hydrolysate. The purine determinations were all carried out with *n*-butanol-diethylene glycol-morpholine as the solvent; for the estimation of pyrimidines *n*-butanol was employed.

† In this experiment the duration of hydrolysis was doubled to 2 hours.

TABLE VIII

*Pancreas Pentose Nucleic Acid; Proportions and Balances**

Compound	Content in nucleic acid	Nitrogen in nucleic acid	N accounted for	Purine N / Pyrimidine N	Mole per mole P	P accounted for	Molar proportions
	per cent	*per cent*	*per cent of nucleic acid N*			*per cent of nucleic acid P*	
Adenine	5.7	2.95	19.2		0.166	16.6	3.6
Guanine	15.5	7.18	46.6		0.402	40.2	8.8
Cytosine	5.8	2.19	14.2		0.205	20.5	4.5
Uracil	1.3	0.32	2.1		0.046	4.6	1.0
Total nucleic acid			82.1	4.0		81.9	

* The nucleic acid preparation contained N 15.4, P 7.9 per cent.

Sugar Component of Pentose Nucleic Acids

Preliminary experiments were carried out on the nature of the pentose liberated by the cleavage of the purine nucleotide moiety of the nucleic acids studied. It was of interest to ascertain the possibility of examining the sugars in minute amounts of hydrolysates by means of filter paper chromatography (20). This proved feasible, particularly when the development method for sugars recently described from this laboratory (21) was employed.

The paper used was Schleicher and Schüll, No. 597. The solvent system consisted of a mixture of 4 volumes of *n*-butanol, 1 volume of ethanol, and 5 volumes of water; the upper organic layer was used for the chromatographic separation which was permitted to proceed for about 12 hours at room temperature. In all experiments, adjacent chromatograms were made with authentic D-ribose and also with D-xylose which is the pentose nearest to ribose in partition behavior.

3 mg. portions of the yeast and pancreas nucleic acids each were hydrolyzed with 0.3 cc. of N H_2SO_4 at 100° for 1 hour. During this time the volume was kept approximately constant by the addition of a few drops of water. For the chromatograms 0.01 cc. portions of the hydrolysates were employed; the drops were neutralized on the paper by exposure to gaseous NH_3. Following the separation the paper strips were treated with *m*-phenylenediamine dihydrochloride as described before (21). The strongly fluorescent spots indicated the presence of one sugar only, which was in all probability D-*ribose*. The R_F values of the sugar components of both yeast and pancreas nucleic acids were 0.30 and coincided completely with the position of authentic ribose. D-Xylose, on the other hand, occupied an unmistakably different position, with an R_F value of 0.24.

Appendix: Characterization of All Nitrogenous Constituents in One Nucleic Acid Sample

In cases in which the scarcity of the available material precludes two separate weighings for the determination of the purines and the pyrimidines respectively, the following procedure may be employed, which involves the liberation of the purines by mild acid hydrolysis and the, at least partial, precipitation of the pyrimidine nucleotides by uranyl acetate in a manner similar to that followed with the purine nucleotides (compare (22)). With regard to the estimation of the purines the procedures are identical with the ones described above, and the figures found have the same significance; the figures for the pyrimidines, however, have at best a semiquantitative value, though the method may be of interest for the characterization of the pyrimidines in very small amounts of nucleic acid.

Between 15 and 25 mg. of the nucleic acid preparation, previously dried in a high vacuum at 60° for 3 hours, were weighed exactly into a small bomb tube, 1.5 cc. of N H_2SO_4 were added, and the sealed tube was heated for 1 hour at 100°. The hydrolysate was brought up to a volume of exactly 2.5 cc. with 0.1 N H_2SO_4, either directly or after adjustment to pH 0.8 to 1, as was described above in the section on the estimation of purines. A portion of this solution (0.3 cc.) served for the purine determinations.

An exact 2.0 cc. aliquot of the solution was placed in a centrifuge cup and adjusted to pH 7 with the help of a mixed indicator. Guanine, which precipitated in part, was centrifuged off and washed once with water. From the united supernatants the nucleotides were precipitated by the addition of a sufficient amount of a saturated (7 per cent) uranyl acetate solution. 10 minutes later the mixture was centrifuged and the supernatant brought to neutrality, at which time a second precipitation occurred. The supernatant was discarded and the united precipitates were dissolved in about 1 cc. of 2 N HCl in order to remove purine traces. The neutralization of this acidic solution brought about the reprecipitation of the uranium salts. The mixture was treated with 1 to 2 drops of the uranyl acetate solution, allowed to stand for 10 minutes, and then centrifuged. The sediment was dissolved or suspended in 0.3 cc. of about 100 per cent formic acid[5] and the solution transferred quantitatively to a bomb tube, two 0.1 cc. portions of formic acid being used for washing. The sealed bomb tube was heated to 175° for 2 hours; the cooled hydrolysis mixture was freed of a white sediment by centrifugation and the supernatant introduced into a 1 cc. volumetric flask. The sediment was washed as often as possible with small amounts of water which served to bring the volume to 1 cc. The chromatography and estimation of the pyrimidines then were carried out in the prescribed fashion.

The total of pyrimidines recovered in this manner was considerably lower than in the procedure discussed above for the quantitative estimation of pyrimidines. The loss was largely attributable to a disappearance of cytosine which in this case, however, was not accompanied by a rise in uracil. The following pyrimidine figures were, for instance, found for yeast ribonucleic acid: cytosine 4.3 and uracil 1.8 per cent, which corresponded to 64 and 78 per cent respectively of the authentic values given in Table V. For the pancreas nucleic acid the figures were cytosine 2.9 and uracil 1.0 per cent, amounting to a recovery of 53 per cent of the cytosine and of 83 per cent of the uracil reported in Table VII.

That this loss in cytosine could not be due to the partial solubility of the uranium salt of cytidylic acid itself was shown in a control experiment with cytidylic acid (Preparation 1). 99.8 mg. of the nucleotide were dissolved in 0.1 N HCl to give a volume of exactly 25 cc. It was found that the entire P contained in 1 cc. aliquots of this solution went into the precipitate produced by uranyl acetate. However, the uranium precipitates thus obtained, when heated at 175° with concentrated formic acid for varying periods, yielded cytosine values that were considerably lower than those given in Table II. The percentages of cytosine found were as follows: after 90 minutes heating 21.4, after 120 minutes 22.7, after 150 minutes 18.4.

---

[5] While the uranium precipitate of cytidylic acid was completely soluble at this state, a small amount of an insoluble residue remained when hydrolysates of pentose nucleic acids were examined.

DISCUSSION

Since this study is in many ways the first of its kind, the discussion may well begin with a brief consideration of its limitations and shortcomings. First of all, it deals solely with the *composition* of nucleic acids; its bearing on the problem of nucleic acid structure is only indirect. Although the recognition of structural principles requires an exact knowledge of the nature and the proportions of all constituents, the findings reported here demand no particular assumption with respect to a specific arrangement of the components or to the type of linkage holding them together. Secondly, it must be understood that all figures presented refer to the hydrolysates only. Groupings that are not cleaved in the course of hydrolysis and compounds that are destroyed during the cleavage of the nucleic acid or that are not liberated at all will, of course, escape detection. The hydrolysis may, on the other hand, lead to the production of artifacts, not present as such in the starting material. These reservations naturally apply to the constituent analysis of all complicated organic substances.

The quality of the nucleic acid preparations also may be responsible for a number of errors. It is known that pentose nucleoproteins in general require a much more drastic treatment for the detachment of the nucleic acid than is the case with the corresponding desoxypentose nucleic acid complexes; and the commercially available preparations of yeast ribonucleic acid probably are badly degraded, as indicated by the numerous purification procedures found in the literature (6) and also by the not infrequently discordant results obtained with different specimens (2). The situation is more favorable with respect to the pentose nucleic acid from pancreas and even more so as regards the desoxypentose nucleic acids which will be discussed in a forthcoming publication. In any event, general principles of composition can already be recognized in the pentose nucleic acids.

That satisfactory methods of hydrolysis are among the most important requisites for the complete characterization of the composition of nucleic acids requires no added emphasis. The liberation of the purines is generally assumed to be achieved by mild acid hydrolysis (compare p. 223 in (1) and also (19)); and this was definitely proved in the present study with purified adenylic and guanylic acids from which adenine and guanine in the respective yields of 95 and 100 per cent of the expected amounts were isolated. The complete release of the pyrimidines presents a much more difficult problem. The method commonly employed, *viz.* prolonged autoclaving with strong mineral acid at a high temperature, must, in the light of the findings reported here, have led to very erroneous conclusions. It has now been found that under these conditions a large part of the cytosine of the nucleic acid is converted to uracil. It was, therefore, necessary to develop a hydrolysis procedure that would permit the attainment of the maximal liberation of constituents without the production of artifacts or of a shift in proportions. Concentrated formic acid was finally chosen for this purpose. This treatment yielded values that were often higher, but in no case lower, than those for total pyrimidine content found with strong mineral acid. Furthermore, since it effected no appreciable conversion of cytosine to uracil, the important question of the presence of uracil as such in the pentose nucleic acids became susceptible of an answer.

The great advantage of the estimation method employed here lies in the fact that it makes possible a complete survey of the distribution of purines and pyrimidines in minute amounts of nucleic acid hydrolysates[6] and that it is capable of distinguishing between the different nitrogenous constituents. Since the total hydrolysate is subjected to a partition between solvents, all components, regardless of their physical properties, have an equal chance of being demonstrated without having to undergo wasteful conversions into derivatives.

The accuracy of the chromatographic separation of the purines and pyrimidines has been discussed in the preceding communication (7). It now remains to consider the faults that could vitiate the analytical results on nucleic acids. If pyrimidines had partly been liberated in the course of the mild hydrolysis employed for the release of purines, slightly too high a value could have resulted for adenine, due to contamination with uracil and, perhaps, also with cytosine. This is, however, unlikely, not only because of the spectroscopic controls that were invariably applied, but also because of the well known stability of the pyrimidine nucleotides. The latter property could, however, be a cause of error in the pyrimidine determinations; this will be taken up later. Another error could have been introduced by the contamination of the pyrimidine fraction with purines that had not been removed completely as the hydrochlorides. This is even less likely; in the chromatographic procedure used, the cytosine fraction could have contained traces of hypoxanthine which, however, probably is not a common nucleic acid constituent and is quite different spectroscopically from cytosine. The absorption maxima of uracil and of adenine, its possible contaminant, are nearer, but since no uracil spot was detected in the corresponding hydrolysates of desoxypentose nucleic acids, contamination with adenine is not likely to have played an important rôle.

---

[6] Even smaller quantities of nucleic acid than were used here could doubtless be employed for the estimations, if the hydrolysates were adjusted to a lesser volume, or if, without volume adjustment, a known amount of a substance were added to the hydrolysates which, serving as a marker, could be separated and estimated quantitatively on the chromatogram.

Before the results presented in this study are compared with those of previous workers, one additional point should be stressed. The inspection of Tables VI and VIII will show that in both nucleic acid preparations a certain proportion (15 to 18 per cent) was not accounted for. Several reasons could be responsible for this deficit. It could, for instance, have resulted from the summation of hydrolysis losses affecting equally the estimations of all the purines and pyrimidines. One observation would seem to favor this assumption. When the total number of gm. atoms of nitrogen found in the hydrolysates is divided by the total number of moles of the four bases in which they are contained (see molar proportions in Tables VI and VIII), the quotients, which indicate the average nitrogen content of each nitrogenous constituent, are in very good agreement with the atomic nitrogen to phosphorus ratio calculated from the analytical values found for the intact nucleic acids. For yeast ribonucleic acid this quotient is 4.1, the atomic N:P ratio 4.2; the corresponding figures for the pancreas nucleic acid are 4.3 and 4.3. This agreement suggests that the hydrolyses did not result in a considerable fractionation.

Another possibility is that the nucleic acids contained small amounts of unidentified components which either were resistant to hydrolysis or gave rise to substances that could not be demonstrated by the chromatographic methods. In this connection, attention may be drawn to the behavior of isolated cytidylic acid toward hydrolysis, discussed above in conjunction with the experimental findings. Several peculiarities exhibited by ribonucleic acids, *e.g.* the lability of the internucleotide linkages toward alkali, cannot be explained on the basis of the currently assigned structures; and these problems will have to be investigated further in connection with the behavior of isolated pyrimidine nucleosides and nucleotides. It will be remembered that dephosphorylation of the nucleic acids appeared complete under the conditions of hydrolysis (Table II); but it is not impossible that a small proportion of differently linked pyrimidines was not liberated by acid hydrolysis. Chromatographic separation studies on the distribution of the constituent nucleotides and other investigations, which are being carried out at present, will perhaps contribute to a decision.

The identification of the sugar component that is associated with the purine moiety of the pentose nucleic acids studied here was attempted, in order to test the possibility of applying a chromatographic microprocedure to the investigation of the carbohydrates present in minute amounts of nucleic acids. Only one sugar was detected in the hydrolysates of the yeast and the pancreas nucleic acids. It was identical in partition behavior with D-ribose, which is in accordance with the original findings of Levene and Jacobs (23).

When the tetranucleotide structure of yeast ribonucleic acid is discussed in the literature (compare, for instance, (3) p. 198), reference is usually made to a publication of Levene (24) that is said to provide the evidence for the occurrence in this nucleic acid of the two purines and the two pyrimidines in equimolecular proportions. Actually, this is far from correct. From 10 gm. of nucleic acid (N 15.2, P 8.6 per cent) 2.0 gm. of adenine picrate and 1.0 gm. of guanine were isolated. The same quantity of nucleic acid yielded cytosine, as what is described as 3.0 gm. of a crude picrate, and uracil whose isolation was reported without any indication of its weight. The nitrogenous bases were, therefore, distributed as follows: adenine 7.1 per cent (0.19 mole per mole of P); guanine, in good agreement with the present findings, 10 per cent (0.24 mole); cytosine (if the crude derivative is considered as cytosine picrate) 9.8 per cent (0.32 mole); no value can be assigned to uracil. The corresponding molar proportions, *viz.* adenine 1.0, guanine 1.3, cytosine (?) 1.7, hardly lend themselves to the formulation of a regular tetranucleotide. The figures assembled in Table VI in the present paper provide, in fact, much better evidence of regularity, but further considerations of this kind should be postponed.

Most of the other evidence is of a more circumstantial nature, *e.g.* the rates of liberation of phosphoric acid (25, 26) and of sugar (26, 27), the calorimetric behavior of yeast nucleic acid (28), the amount of total purine nitrogen liberated (19), etc. A discussion of the analytical data submitted in a preliminary form by Loring *et al.* (29) should, in view of a recent note (30), await the presentation of experimental details.

The pentose nucleic acid of pancreas, while much investigated, has not had as important a part as yeast nucleic acid in the development of the conception of nucleic acid structure, though its peculiar composition early served to draw attention to the far from simple problems involved. It was suspected of not fitting into the pattern of a regular tetranucleotide and was in turn formulated as a guanylic acid complex of ribonucleic acid and as a hexa- or pentanucleotide. Hammarsten (31) assumed a guanine to adenine ratio of 3:1, Steudel (32) of 4:1, Levene and Jorpes (12) found in different preparations 3.3 and 4.6 times as much guanine as adenine, and Jorpes (11) twice as much guanine as adenine. Since some of the guanylic acid present in this material appears to be bound rather feebly, it is probable that different methods of isolation led to products of somewhat varying composition, quite apart from the uncertainties inherent in the estimation methods.

It will be seen that the findings summarized in Table VIII correspond to about 4 molecules of adenine and 5 of cytosine per 10 molecules of guanine. The ratio of purine to pyrimidine nitrogen was very high. Since pancreas is rich in ribonuclease which appears to act preferentially on the pyrimidine nucleotide portion of ribonucleic acids (33, 34), it is not impossible that some pyrimidine was liberated in the course of the preparation of the material.

One more point remains to be considered, namely the presence of uracil in the pentose nucleic acids. That cytosine is converted to uracil with relative ease has long been known (35); and in view of the extremely energetic methods commonly used for hydrolysis it is natural that the possibility of uracil being an artifact has formed the subject of lively controversies. Kowalevsky (36), in an investigation of yeast ribonucleic acid carried out in Steudel's laboratory, attempted to demonstrate the exclusive presence of three nitrogenous bases which she claimed to occur in the following molar proportions: adenine 1, guanine 1.6, cytosine 1.1. While the concentration of uracil in the hydrolysates of both pentose nucleic acids examined in the present study is very low, there is every reason to believe that it is a genuine constituent of the preparations. To what extent, however, the effect of deaminases and of other enzymes acting on the nucleic acids during their isolation may contribute to their final composition is a problem that will have to be considered separately.

The authors wish to thank Miss Ruth Doniger and Mrs. Charlotte Green for excellent assistance and Mr. W. Saschek and Miss R. Rother for the microanalyses.

SUMMARY

The methods for the separation and estimation of adenine, guanine, cytosine, and uracil in minute amounts, described in the preceding communication, were applied to a study of the distribution of these nitrogenous constituents in hydrolysates of the ribonucleic acids of yeast and of pancreas. Since it was shown that under the customary conditions of hydrolysis with strong mineral acid cytosine was largely converted to uracil, concentrated formic acid was chosen as the hydrolyzing agent.

The presentation of detailed methods for the preparation of the hydrolysates and the estimation of the individual components is followed by a consideration of the proportions in which the purines and pyrimidines were found in the hydrolysates. A procedure, permitting the characterization of the nitrogenous constituents in very small quantities of nucleic acid,

and the application of chromatography on filter paper to the identification of the carbohydrate components of nucleic acids likewise are described.

The paper, which includes studies of the hydrolysis behavior of isolated nucleotides, concludes with a critical discussion of the findings.

BIBLIOGRAPHY

1. Levene, P. A., and Bass, L. W., Nucleic acids, American Chemical Society monograph series, New York (1931).
2. Gulland, J. M., Barker, G. R., and Jordan, D. O., Annual review of biochemistry, Stanford University, **14**, 175 (1945).
3. Tipson, R. S., in Pigman, W. W., and Wolfrom, M. L., Advances in carbohydrate chemistry, New York, **1**, 193 (1945).
4. Nucleic acid, Symposia of the Society for Experimental Biology, Cambridge, **1** (1947).
5. Chargaff, E., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **12**, 28 (1947).
6. Chargaff, E., and Vischer, E., Annual review of biochemistry, Stanford University, **17**, 201 (1948).
7. Vischer, E., and Chargaff, E., *J. Biol. Chem.*, **176**, 703 (1948).
8. Fletcher, W. E., Gulland, J. M., and Jordan, D. O., *J. Chem. Soc.*, 30 (1944).
9. Chargaff, E., and Zamenhof, S., *J. Biol. Chem.*, **173**, 327 (1948).
10. Jorpes, E., *Acta med. Scand.*, **68**, 503 (1928).
11. Jorpes, E., *Biochem. J.*, **28**, 2102 (1934).
12. Levene, P. A., and Jorpes, E., *J. Biol. Chem.*, **86**, 389 (1930).
13. Buell, M. V., *J. Biol. Chem.*, **150**, 389 (1943).
14. Steudel, H., and Peiser, E., *Z. physiol. Chem.*, **114**, 201 (1921); **120**, 292 (1922).
15. Levene, P. A., *J. Biol. Chem.*, **41**, 19 (1920).
16. Levene, P. A., *J. Biol. Chem.*, **41**, 483 (1920).
17. Hunter, G., and Hlynka, I., *Biochem. J.*, **31**, 486 (1937).
18. Levene, P. A., *Z. physiol. Chem.*, **39**, 4 (1903).
19. Graff, S., and Maculla, A., *J. Biol. Chem.*, **110**, 71 (1935).
20. Partridge, S. M., *Nature*, **158**, 270 (1946).
21. Chargaff, E., Levine, C., and Green, C., *J. Biol. Chem.*, **175**, 67 (1948).
22. Kerr, S. E., *J. Biol. Chem.*, **132**, 147 (1940); **145**, 647 (1942).
23. Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, **42**, 2102, 2469, 3247 (1909).
24. Levene, P. A., *Biochem. Z.*, **17**, 120 (1909).
25. Jones, W., Nucleic acids, Monographs on biochemistry, 2nd edition, London and New York, 40 (1920).
26. Kobayashi, Y., *J. Biochem.*, Japan, **15**, 261 (1932).
27. Hoffman, W. S., *J. Biol. Chem.*, **73**, 15 (1927).
28. Ellinghaus, J., *Z. physiol. Chem.*, **164**, 308 (1927).
29. Loring, H. S., Ordway, G. L., Roll, P. M., and Pierce, J. G., *Federation Proc.*, **6**, 510 (1947).
30. Loring, H. S., and Fairley, J. L., Jr., *J. Biol. Chem.*, **172**, 843 (1948).
31. Hammarsten, E., *Z. physiol. Chem.*, **109**, 141 (1920).
32. Steudel, H., *Z. physiol. Chem.*, **241**, 84 (1936).
33. Loring, H. S., Carpenter, F. H., and Roll, P. M., *J. Biol. Chem.*, **169**, 601 (1947).
34. Schmidt, G., Cubiles, R., Swartz, B. H., and Thannhauser, S. J., *J. Biol. Chem.*, **170**, 759 (1947).
35. Wheeler, H. L., and Johnson, T. B., *Am. Chem. J.*, **29**, 492 (1903).
36. Kowalevsky, K., *Z. physiol. Chem.*, **69**, 240 (1910).

A NEW PREPARATION OF CRYSTALLINE ANTERIOR PITUITARY GROWTH HORMONE*

By ALFRED E. WILHELMI, JACOB B. FISHMAN, and JANE A. RUSSELL

With the Technical Assistance of Marjorie Cappiello

(*From the Department of Physiological Chemistry, Yale University, New Haven*)

(Received for publication, July 21, 1948)

The isolation and identification of the anterior pituitary growth hormone as a pure protein were first achieved by Li, Evans, and Simpson (1, 2) in 1944–45. Their method is a beautiful example of the classical procedures of protein fractionation by the controlled use of salt and of hydrogen ion concentrations. It owes its distinction to the proof that the growth-promoting activity of anterior pituitary extracts, first demonstrated by Evans and Long (3), is attributable to an individual protein. The disadvantages of the method lie in the somewhat low extraction efficiency from acetone powders of the anterior pituitary glands, in the tedium of the many repeated steps of the process, in the time consumed, in the losses inevitably associated with repeated manipulations, and in the resulting low yields of product (of the order of 60 mg. per kilo of fresh anterior pituitary glands). The method attains its objective of isolating pure growth hormone, and it secures information about the properties of the protein that is essential in the development of an improved method, but is impractical for obtaining the hormone in the quantities required for extensive experimental and clinical investigation.

It has been found that a fractionation of calcium hydroxide extracts of fresh bovine anterior pituitary glands, by means of ethanol at low temperatures along the lines successfully exploited by Cohn and his colleagues (4) in the separation of the plasma proteins, yields an abundance of crude fractions with high growth-promoting activity. Re-solution of these active fractions in dilute potassium chloride, followed by removal of the bulk of impurities in a precipitate formed at pH 5.0 and by a fractional precipitation with ethanol, starting at pH 8.5 to 8.7, yields a crystalline protein, electrophoretically homogeneous, which from its biological activity and other properties is identified as the anterior pituitary growth hormone. The yields of the crude primary fractions average about 33 gm. per kilo of fresh glands. The yields of the purification procedure lie between 8 and 16 per cent. On this basis, the new method can be expected to produce quantities

---

* The work reported in this paper is being done under a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

of pure or nearly pure crystalline growth hormone of the order of 3 gm. per kilo of fresh glands.

Methods

The growth-promoting action of the various fractions was measured by their effects on the body weight of male hypophysectomized rats,[1] of about 100 gm. body weight, brought about by the daily intraperitoneal injection of solutions of the test material in normal saline for 10 days. In most instances the effects on growth were checked by measurements of the width of the proximal epiphyseal cartilage of the tibia, as described by Evans, Simpson, Marx, and Kibrick (5). Adrenocorticotropic activity was measured by the method of Sayers, Sayers, and Woodbury (6). Glycostatic activity was measured by the effects of two intraperitoneal injections of the test substance in normal saline solution on the level of the muscle glycogen of 200 to 250 gm. hypophysectomized male rats fasted for 24 hours (7). All of the experimental animals were maintained on a diet of laboratory chow in a room at a constant temperature of 25.5°.

Determinations of pH were made with the glass electrode.

Method of Isolation—The anterior lobes were carefully dissected from frozen bovine pituitary glands and were stored in a freezer until required. Small lots of 300 to 500 gm. were used for the individual runs.

The glands are prepared for extraction by thawing them slightly, mixing the separated glands with coarsely ground solid carbon dioxide, and grinding them to a fine powder by two or three passes, with successively closer plate settings, through a Straub dry food grinder previously chilled with solid carbon dioxide. The carbon dioxide is allowed to evaporate, leaving a cold pink mush of the ground glands.

All subsequent operations are carried out in a cold room at 0–5°.

The ground glands, free of carbon dioxide, are suspended in dilute calcium hydroxide solution, pH 11.5, and stirred mechanically for 24 hours. 2 liters of calcium hydroxide solution are used for 300 to 350 gm. of fresh frozen glands. During the early hours of stirring, the pH of the mixture must be maintained at 11.5 by occasional additions of solid calcium hydroxide. The pH becomes steady after 3 or 4 hours, and, in the conditions in our cold room, it is usually not less than pH 11.0 at the end of 24 hours stirring.

The pH of the mixture is adjusted to 8.5 to 8.7 by bubbling in carbon dioxide gas, care being taken not to overrun, and the mixture is allowed to settle overnight. It is thereafter centrifuged, the opalescent pink supernatant solution is decanted, and the residue is discarded.

---

[1] The hypophysectomized rats used in the growth tests were purchased from Hormone Assay, Inc., of Chicago, Illinois. The condition, survival, and uniformity of response of these animals were excellent.

To the supernatant solution, rapidly stirred, a calculated volume of a mixture of equal parts of 95 per cent ethanol and water (1:1 ethanol) is added dropwise, at a rate of about 60 ml. per hour, until the concentration of ethanol in the mixture is 12 per cent. The precipitate that forms is centrifuged off, Fraction A.

To the 12 per cent ethanol supernatant solution, vigorously stirred, 1:1 ethanol is added dropwise until the ethanol concentration is 24 per cent. The precipitate is centrifuged off, Fraction B.

The 24 per cent ethanol supernatant solution is adjusted to pH 6.8 with 4 N hydrochloric acid, and the resulting precipitate centrifuged off, Fraction C.

The supernatant solution is adjusted to pH 4.6 with 4 N hydrochloric acid, and the resulting precipitate centrifuged off, Fraction D.

To the 24 per cent ethanol supernatant solution, pH 4.6, vigorously stirred, 95 per cent ethanol is added dropwise until the ethanol concentration of the mixture is 40 per cent. The resulting precipitate is centrifuged off, Fraction E.

The clear pink supernatant solution is discarded.

Fractions A to E are each suspended in distilled water and lyophilized. They constitute the crude primary fractions. Of these, Fractions A, B, and C contain nearly all of the growth activity, and these fractions may be purified in the following steps.

A 0.5 per cent solution of Fraction A, B, or C in 0.1 N potassium chloride solution is made with the aid of 1 N potassium hydroxide, added until the pH of the mixture is 11.0. Solution is usually complete; if it is not, the solution is cleared by centrifuging.

The pH of the solution is adjusted to 5.0 with 4 N hydrochloric acid. The resulting precipitate is centrifuged off, suspended in one-half the original volume of 0.1 N potassium chloride solution, and saved.

The supernatant solution, water-clear and usually nearly colorless, is adjusted to pH 8.5 to 8.7 with 1 N potassium hydroxide, and a calculated volume of 1:1 ethanol is added dropwise (rate, 30 ml. per hour) to the rapidly stirred solution until the ethanol concentration of the mixture is 5 per cent. The resulting precipitate, which exhibits some crystals on microscopic examination, is centrifuged off and saved.

To the water-clear 5 per cent ethanol supernatant solution, vigorously stirred, the calculated volume of 1:1 ethanol is added dropwise until the ethanol concentration of the mixture is 20 per cent. A crystalline precipitate forms slowly and steadily throughout the period of addition, and the process appears to be complete by the end of the addition. The crystalline precipitate is pure, or nearly pure growth hormone. It is collected by centrifugation, suspended in distilled water, dialyzed against distilled water until salt-free, and lyophilized. A photograph of the crystalline product is presented in Fig. 1.

The precipitate, pH 5.0, together with the 5 per cent ethanol precipitate, may be redissolved in one-half the original volume of 0.1 N potassium chloride solution with the aid of 1 N potassium hydroxide and put through the procedure once more to yield additional crystalline product. The products obtained in a second and third rework of the precipitate at pH 5.0 are in small yield and are not homogeneous.

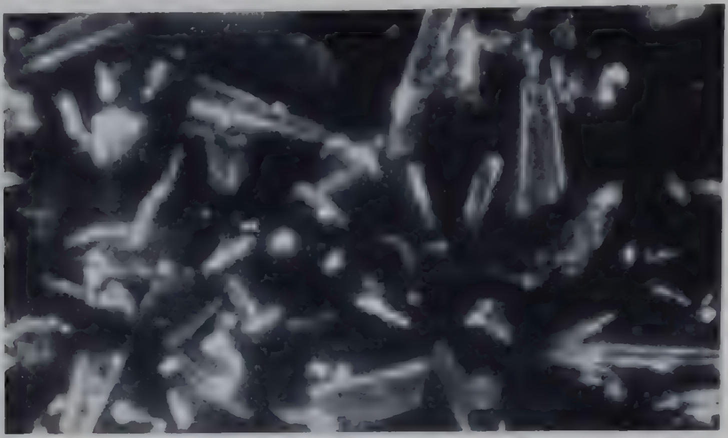


Fig. 1. Growth hormone crystals, photographed in their mother liquor. Dark-field; magnification × 430.

TABLE I

Yields of Total Solids and of Crude Fractions A to E in Nine Extractions of Fresh Beef Anterior Pituitary Glands

Experiment No.	Glands, fresh weight	Solids in extract		Fractions					
				A	B	C	D	E	A + B + C
	gm.	*gm.*	*gm. per kg.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm. per kg.*
12	304			0.6	1.9	4.0	1.2	0.4	21.7
46	304	23.5	77.3	4.6	2.3	4.6	3.5	1.2	38.3
62	305	24.9	81.6	16.6*			1.4		55.3
82	335	32.1	95.8	9.5	3.4	2.3	2.6	0.9	45.2
115	350	23.8	68.0	4.5	8.6	2.8	1.9	0.6	45.3
143	350	14.6	41.7	2.9	1.3	0.4	1.1		13.1
168	500	28.1	56.2	10.3	1.9	0.2	1.9		24.8
194	430	26.4	61.4	5.8	2.0	0.6	2.0		19.6
218	382	23.5	61.5	8.7*			0.3		22.7

* Fractions A, B, and C not separated.

Yields

A summary of the yields of total solids in the crude extracts of several separate runs and of the amounts of Fractions A to E is presented in Table I. The total solids of the pH 8.5 to 8.7 extract, measured by lyophilizing an aliquot of the extract, average nearly 69 gm. per kilo of fresh glands. At an average of 12 per cent of nitrogen, this amounts to 8.38 gm. of nitrogen per kilo of fresh glands. Li, Evans, and Simpson (2) report a yield of 4.37 gm. of nitrogen per kilo of fresh glands in one whole extract of an acetone powder. It seems probable that active material may be more readily and completely extracted from the finely ground fresh glands than from the acetone powder.

The yields of Fractions A to E vary somewhat from run to run, and the distribution of material, especially between Fractions A, B, and C, is also irregular. This may be due in part to differences between different lots of glands, and in part to the effects of evaporation from open vessels, during vigorous stirring in a room in which a strong flow of cold air is maintained, on the final ethanol concentration attained at each step. There is also a slow drift of pH from its starting point of 8.5 to 8.7 to about 7.5 to 7.7 at the end of the second ethanol addition, and this may influence the distribution of material between Fractions A and B. More consistent yields of material in each fraction might be attained by carrying out the procedure in closed vessels. The combined yield of Fractions A, B, and C is about 33 gm. per kilo of fresh glands. Slightly less than one-half of the total solids in the extract is collected in the five crude fractions. The final supernatant solution has not yet been explored for its content of other kinds of pituitary hormone activity.

TABLE II

Summary of Yields of Crystalline Growth Hormone from Experiment 194

	gm.	gm.
Fresh glands extracted		430
Fraction A		5.8
Crystals (1st run)	0.939	
" (2nd ")	0.177	
Fractions B + C		2.5
Crystals (1st run)	0.159	
" (2nd ")	0.072	
Total crystals	1.347	

Yield, 2.99 gm. of crystals per kilo of fresh glands.

The yields of crystalline material obtained from Fractions A, B, and C of one run are outlined in Table II. In this instance, the yield is about 17 per cent of the crude starting material. This step of the procedure was developed during attempts to purify the crude crystalline preparations described in a preliminary report of the method (8). In these trial procedures, yields of electrophoretically homogeneous material of the order of 8 to 20 per cent of the starting material were obtained. It may therefore not be unreasonable to expect yields of crystalline material by the new and simpler procedure to be of the order of 3 gm. per kilo of fresh anterior pituitary glands.

Electrophoretic Analysis

Because of the uncertainties of the growth assay and the fact that Fractions A, B, and C already exhibit an order of growth activity such that it is difficult to discriminate them biologically from more highly purified materials, the progress of purification has been followed electrophoretically. The Tiselius apparatus employed is equipped with the Longsworth modification of the Philpot scanning device. For many of the routine observations a buffer of the composition, glycine 0.1 M, sodium hydroxide 0.67 M, sodium chloride 0.1 M, pH 10.0, ionic strength 0.17, is employed, since it was found that good clear solutions of 1 per cent protein concentration were most easily obtained in this buffer, especially with the cruder fractions. In Fig. 2, *a* are seen the electrophoretic patterns in glycine buffer pH 10 of the

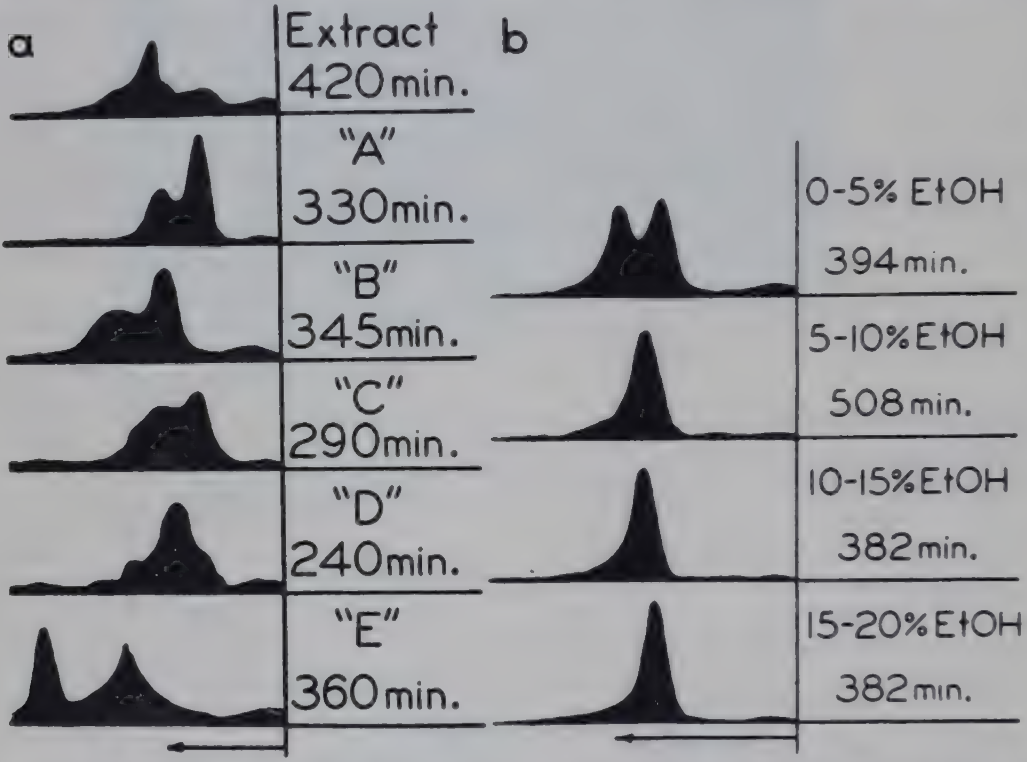


FIG. 2. (*a*) Tracings of the electrophoretic patterns of the crude extract and of the five primary fractions. The large slow peaks in Fractions A and B are identifiable by their mobilities with the growth hormone. (*b*) Tracings of the electrophoretic patterns of successive fractions obtained during the fractional crystallization of the growth hormone from KCl solution with ethanol. All of the tracings are of ascending boundaries. Glycine buffer pH 10.0, ionic strength 0.17, temperature 4°, current 17 milliamperes.

whole extract and of the five crude fractions. The large slow peak seen in Fractions A, B, and C has a mobility characteristic of that of the growth hormone. The corresponding peaks in Fractions D and E comprise a relatively small proportion of the areas of the patterns. This is in good accord with the biological data summarized below. The different patterns of Fractions A, B, and C, taken together with the fact that a colored contaminant of the fractions increases in amount from Fraction A to C, seem to justify taking these fractions separately, as described. In one run these fractions were combined by making a straightforward addition of 1:1 ethanol to a concentration of 24 per cent of ethanol. Since the final purification steps had not been developed at that time, it cannot be said whether an "ABC" fraction would be as easily susceptible of purification as the separate fractions are. This point will be examined, since a considerable economy of effort can be made if the first three fractions can be taken as one.

The advantage of the final fractional precipitation with ethanol is illustrated in Fig. 2, *b*, which is taken from an experiment in which separate precipitates were collected at 5, 10, 15, and 20 per cent of ethanol. Most of the impurity carried over from the precipitation at pH 5.0 appears to be removed at the lowest concentration of ethanol. There is no significant difference between the other three fractions, which are uniformly crystalline. They are therefore taken as one fraction in the routine version of the method.

The electrophoretic patterns of a crystalline preparation of growth hormone in glycine buffer at pH 10 and in acetate buffer at pH 4 are illustrated in Fig. 3. The material appears to be homogeneous, and the mobilities observed at each hydrogen ion concentration correspond closely with the mobilities observed by Li, Evans, and Simpson (2) and by Li[2] for their preparation of pure growth hormone.

This same preparation is being examined for us in the ultracentrifuge by Dr. Emil L. Smith of the University of Utah School of Medicine. In glycine buffer at pH 9.4 and at protein concentrations of 1 and 0.5 per cent, only a single sedimenting boundary is observed, and the rate of sedimentation is of an order indicating a molecular weight of about 49,000 for the material. A more complete ultracentrifugal analysis will be reported at another time, but this preliminary information affords additional evidence of the purity of the crystalline product and of its identity with the pure growth hormone isolated by Li, Evans, and Simpson (2).

Biological Activity

The observed effects of the crude Fractions A to E upon growth and upon the width of the proximal epiphyseal cartilage of the tibia in hypophysecto-

[2] Li, C. H., personal communication.

mized male rats are summarized in Table III. It will be seen that Fractions A, B, and C bring about growth increments of 2 gm. or more per day at a dose level of 100 γ per day. Since the relationship between dose and response is a logarithmic one, these fractions already exhibit unit activity; that is, they can be expected to bring about growth increments of 1 gm. per day at dose levels of 10 γ per day. A full dress assay in an elaborate experimental design is therefore necessary to distinguish the growth potency of these three fractions from that of the crystalline hormone. Fractions D

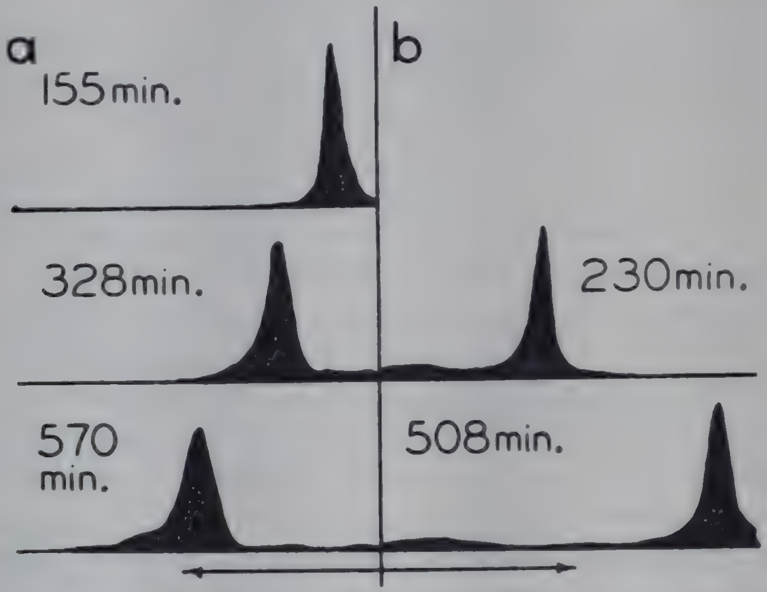


FIG. 3. Tracings of the ascending boundaries observed during the electrophoresis of crystalline growth hormone. (*a*) Glycine buffer pH 10.0, ionic strength 0.17. (*b*) Acetate buffer pH 4.1, ionic strength 0.13. Temperature 4°, current 17 milliamperes.

TABLE III

Effect of Crude Primary Fractions upon Growth and Width of Tibial Epiphyseal Cartilage in Hypophysectomized Rats

Fraction No.	Daily dose	10 day increase in	
		Body weight	Epiphyseal width
	mg.	*gm.*	μ
194-A	0.10	28.3	204
143-B	0.01	8.3	102
12-B	0.10	23.0	
12-C	0.10	21.0	
168-D	0.10	7.6	55
115-D	1.00	18.3	198
115-E	1.00	4.6	75

Each figure is the mean of three or four rats.

and E are much less active, which accords with the electrophoretic evidence that an element identifiable with the growth hormone forms only a minor part of the total electrophoretic pattern. In general, the effects of the different fractions in the different doses upon the widths of the tibial epiphyses correspond very well with their relative effects upon growth.

The growth-promoting effects of the crystalline hormone are summarized in Table IV. Fractions 198-A, 198-B, and 198-C were obtained with 0 to 5, 5 to 10, and 10 to 15 per cent of ethanol respectively. The first of these fractions, as one might expect from the electrophoretic patterns of Fig. 2, *b*, appears to be less active than the others. At the dose level of 20 γ daily, the crystalline fractions exhibit both growth and cartilage effects comparable to those observed with the pure growth hormone of Li, Evans, and Simpson (2).

TABLE IV

Effect of Purified Growth Hormone on Growth and Width of Tibial Epiphyseal Cartilage in Hypophysectomized Rats

Fraction No.	Daily dose	10 day increase in	
		Body weight	Epiphyseal width
	mg.	*gm.*	μ
198-A	0.02	12.3	119
198-B	0.02	16.6	159
198-C	0.02	14.3	150
202-3-BCD	0.02	15.8	180
	0.10	25.0	207

Each figure is the mean of three or four rats.

The crystalline material has been tested for adrenocorticotropic activity by the method of Sayers, Sayers, and Woodbury (6). A dose of 0.5 mg. per 100 gm. of body weight, injected intravenously into each of four 1 day-hypophysectomized rats immediately after the removal of one adrenal gland, produced in 1 hour an average fall in the concentration of ascorbic acid of only 23 mg. per cent in the remaining adrenal gland as compared with that of the control gland. This corresponds to the effect of 0.2 to 0.3 γ of purified adrenocorticotropic hormone, a negligible contamination of the order of 4 to 6 parts in 10,000.

Complete information is not yet available on the other kinds of pituitary hormone activity present in the crystalline growth hormone. A detailed assay of a cruder crystalline preparation made by the old procedure (8), which showed two components electrophoretically, yielded the following results:[3] (*a*) adrenocorticotropic hormone, less than 1.25 per cent of Armour

---

[3] We are grateful to the late Dr. Fred C. Koch, to Dr. Paul L. Munson, and to Mr. Irby Bunding of the Armour Research Laboratories, for their kindness in conducting these assays for us.

LA1A standard; (*b*) prolactin, less than 0.1 per cent of Armour standard (16 international units per mg.); (*c*) gonadotropic, negligible effects on uterine and ovarian weights of hypophysectomized female rats in 4 days at dose levels of 0.4 and 0.8 mg. per day; (*d*) thyrotropic hormone, 7.2 per cent of Armour standard or about 0.1 Evans chick unit per mg.; (*e*) pressor activity, less than 0.01 U. S. P. reference posterior pituitary units per mg.; (*f*) oxytocic activity, less than 0.004 U. S. P. reference posterior pituitary units per mg. This crude material is therefore relatively free of other kinds of anterior and posterior pituitary hormone activities, and it is to be expected that the crystalline hormone, which satisfied at least three of the criteria for purity, will prove to be cleaner in these respects.

All of the active growth hormone preparations examined so far, including the purest crystalline preparations, have exhibited excellent glycostatic activity, which has become more intense with increasing degree of purification. A detailed account of the experiments will be given elsewhere, but it may be said, on the basis of the present observations, that the activity of the anterior pituitary gland in maintaining normal levels of muscle glycogen in the 24 hour fasted hypophysectomized rat, first observed by Russell (7), appears to be a property of the growth hormone.

Work is in progress on a detailed chemical and physicochemical study of the crystalline growth hormone prepared by the present method, in order to compare the properties of this material with those described by Li *et al.* (2, 9, 10) for their pure growth hormone.

Attention has so far been concentrated on developing the new method for isolating growth hormone. It is hoped, however, that the principles and procedure of the new method may be applied to the efficient isolation of other hormones of the anterior pituitary gland.

SUMMARY

A new method is described for the preparation of crystalline growth hormone from fresh bovine anterior pituitary glands. The method depends upon the fractionation with ethanol at low temperatures of a calcium hydroxide extract of the ground, fresh glands. The crude fractions obtained at this stage are purified by an isoelectric precipitation of contaminating material from dilute salt solution, followed by a fractional crystallization with ethanol. The yields of the crystalline product, which is electrophoretically and ultracentrifugally homogeneous, are of the order of 3 gm. per kilo of fresh glands. The crystals have the expected effects upon body growth and upon the width of the proximal epiphyseal cartilage of the tibia in hypophysectomized rats. They are also the most active glycostatic hormone preparations yet isolated; so that it appears that glycostatic activity is one of the properties of the growth hormone.

BIBLIOGRAPHY

1. Li, C. H., and Evans, H. M., *Science*, **99**, 183 (1944).
2. Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, **159**, 353 (1945).
3. Evans, H. M., and Long, J. A., *Anat. Rec.*, **21**, 62 (1921).
4. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, **68**, 459 (1946).
5. Evans, H. M., Simpson, M. E., Marx, W., and Kibrick, E., *Endocrinology*, **32**, 13 (1943).
6. Sayers, M. A., Sayers, G., and Woodbury, L. A., *Endocrinology*, **42**, 379 (1948).
7. Russell, J. A., *Endocrinology*, **22**, 80 (1938).
8. Fishman, J. B., Wilhelmi, A. E., and Russell, J. A., *Science*, **106**, 402 (1947).
9. Li, C. H., *J. Phys. and Colloid Chem.*, **51**, 218 (1947).
10. Franklin, A. L., Li, C. H., and Dunn, M. S., *J. Biol. Chem.*, **169**, 515 (1947).

NOTES ON MYOGLOBIN PREPARATION AND IRON CONTENT

By WILLIAM J. BOWEN

(*From the Laboratory of Physical Biology, National Institute of Health, Bethesda, Maryland*)

(Received for publication, June 22, 1948)

Since the first description by Theorell (11) of a process of isolating and purifying myoglobin from horse heart by crystallization, several workers have succeeded in preparing myoglobin by Theorell's procedure. Roche and Vieil (8) and Rossi and Aragona (9) have described methods less arduous than Theorell's. Their methods, however, sacrifice yield to gain simplicity.

We have developed a modification of Theorell's procedure for preparing horse heart myoglobin in which the more than twenty steps described by him are reduced to about ten with no sacrifice of yield. During the work, difficulty was experienced in attaining purity of the preparations based on the value for the iron content, 0.345 per cent, reported by Theorell (11) and others. It became apparent that the value 0.345 per cent iron is greater than the value computed from the ratio in myoglobin of iron content (1 atom per molecule) and the molecular weight. The results of analyses of our preparations indicate that the value computed from the molecular weight derived from sedimentation and diffusion constants can be used as a criterion of purity of myoglobin.

Preparation

At a slaughter-house, horse hearts are removed as soon as possible and perfused through the coronary arteries with tepid 0.85 per cent NaCl solution (made on the site with warm tap water) until the solution emerging from the veins is colorless. About 15 liters of solution are used per heart. The auricles and as much fat and connective tissue as practical are trimmed away from the ventricles. In the laboratory the work is carried out at 1–5° as far as possible. The ventricular flesh is ground by an electrically driven meat chopper, and the ground meat is mixed with a chilled volume of water equal in ml. to the weight of the meat in gm. The mixture is stirred well and left in the cold overnight.

The following morning the extract is separated by centrifuging at 1000*g* or more. The extract is then partially purified by making the solution 3 M (50 per cent of saturation) with ammonium sulfate and separating the precipitate thus salted out by either centrifugation (1000*g*) or filtration. The supernatant solution is put into cellophane sausage bags 3 or 4 inches in diameter, which are then immersed in a chilled bath of saturated ammonium sulfate that contains an excess of the salt and is adjusted to pH 7.1 to 7.3 with NH_4OH. The solution outside the bag is stirred with an electric stirrer for 24 hours.

Crystals of myoglobin appear in a day or so gathered in typical clusters (11), the form of which resembles sheaves of grain. The clusters increase in size for 2 or 3 weeks and reach lengths up to 1 ml. The myoglobin can be left in the baths indefinitely at about 5°. (We have stored it thus for 1 year.) To isolate the crystals from the amorphous impurities, the contents of the dialysis bag are centrifuged at 1000*g* for 10 minutes. The supernatant solution with as much of the amorphous material as possible is aspirated from the layer of crystals. The remaining precipitate of crystals and amorphous matter is resuspended in saturated ammonium sulfate[1] and centrifuged at 200*g* to 300*g* for 5 minutes. The supernatant solution is again removed by aspiration. The process of resuspension, centrifugation, and aspiration is repeated three times. The precipitate of crystals is next suspended in saturated ammonium sulfate[1] in a 500 ml. graduated cylinder. After 24 hours the larger crystals have settled to the bottom and the solution above them is removed by aspiration. This process of suspension, settling, and aspiration in the cylinder is carried out six times.

After the final washing, the ammonium sulfate is diluted until the myoglobin is dissolved. The solution is then dialyzed in a cellophane bag, 0.75 to 1.0 inch in diameter, in the cold against running distilled water for 24 hours or until a portion of the extract gives no indication of sulfate when tested with 10 per cent barium chloride. The dialysis is carried out on a rocker arm with a bubble in the bag to assure mixing. When the solution is removed from the bag, it contains a light precipitate which is apparently of no consequence and is removed by filtration. The solution of myoglobin thus obtained can be stored indefinitely in a refrigerator if a drop of chloroform is added.

Iron Content

In his original publication, Theorell (11) presented four determinations of iron in horse heart myoglobin which averaged 0.345 per cent. Later, Theorell and de Duve (12) obtained 0.340 per cent in human myoglobin. Either of these values is sometimes claimed to be the iron content of hemoglobin (4, 5, 11), and since myoglobin, like hemoglobin, contains 1 iron atom per unit of heme, it has been accepted as the iron content of myoglobin. Millikan (5) gives 0.345 per cent in a review of research on myoglobin, and Rossi and Aragona (9) state that they obtained this proportion repeatedly. Drabkin (4) found 0.340 ± 0.002 per cent. When used to calculate the molecular weight of myoglobin, 0.345 per cent iron gives 16,186. The value 0.340 gives 16,424.

---

[1] In order to lessen the possibility of contaminating the myoglobin with iron contained as an impurity in ammonium sulfate, the saturated solution was filtered after standing several weeks and the iron in it had precipitated.

The molecular weight of horse heart myoglobin has been determined by five methods. These are listed in Table I, together with the calculated values for iron content on the assumption of 1 atom of iron per molecule. It is to be noted that the results are consistently lower than that obtained by Theorell. Zinoffsky (14) found, by painstaking methods, that the iron content of horse hemoglobin is also less than 0.345 per cent; he obtained 0.335 per cent. Valer (13), working with care equal to Zinoffsky's, obtained 0.330 per cent.

TABLE 1

Molecular Weight of Myoglobin from Literature and Calculated Iron Content for Each Observation

Mol. wt.	Iron calculated from mol. wt.	Method of determination of mol. wt.	Observer and reference No.
	per cent		
16,850	0.331	Osmotic pressure	Roche and Vieil (8)
17,200	0.325	Sedimentation and diffusion	Polson (6)
17,500	0.319	Sedimentation-equilibrium	" (10)
17,534*	0.318	Bergmann and Niemann (1)	Roche and Derrien (7)
17,600	0.317	Rule of simple multiples	Pedersen (10)

* Roche and Derrien actually give 16,934, but they neglected to add the value of the molecular weight of 1 heme molecule (600) to the weight of the globin portion determined by them.

The molecular weight 17,500 (Table I), according to Pedersen (10), is the mean of four values obtained from determinations of the sedimentation equilibrium by Polson. Polson (6) also determined the molecular weight of myoglobin from the same preparation from sedimentation and diffusion constants, with correction for particle size. In this instance he obtained 17,200. Technical difficulties prevent the exact measurement of these values by sedimentation and diffusion methods and the publications cited give few of the data necessary for statistical evaluation. Polson (6) presents five experimental diffusion constants taken on two concentrations of myoglobin, which vary less than 1.1 per cent from the mean; however, according to Edsall (3), Svedberg has estimated the probable error in diffusion and sedimentation constants as 2 to 3 per cent and in calculated molecular weights as 5 to 10 per cent.

We have analyzed several preparations of myoglobin, prepared as described above, for iron. These analyses were made by the method described by Delory (2), with α,α'-dipyridyl as an indicator. To establish the accuracy of the method, a statistical analysis of its reliability was made. First, twelve standards, to each of which were added 25 γ of iron per 10 ml., were analyzed. These gave an average iron content of 25.02 γ (σ = 0.80; coefficient of variation = 3.2 per cent). Secondly, nine samples of 1 ml. each from a single solution of horse heart myoglobin known to be only partially pure were analyzed for iron content and dry weight. The iron ranged from 6.00 to 6.19 γ, with an average value of 6.10 (σ = 0.2; coefficient of variation = 3.3 per cent). The dry weights ranged from 1.97 to 2.10 mg. per ml. and averaged 2.03 mg. (σ = 0.14; coefficient of variation = 6 per cent).

Finally eight preparations of myoglobin of different concentrations were analyzed by this method. The determinations of iron in them were 0.313, 0.317, 0.305, 0.322, 0.352, 0.325, 0.340, and 0.306 per cent, average 0.323 (σ = 0.043; coefficient of variation = 13.3 per cent).

The possibility exists that this value for the iron content of myoglobin is affected by loss of either the heme or globin of denatured molecules. The solubility and stability of globin from myoglobin are unknown for the conditions of the preparation; however, from knowledge of the relative stabilities of globin and heme of hemoglobin, one would expect the heme to be more stable than the globin. If this obtains for myoglobin, denaturation would increase the iron content rather than decrease it, and would give values equal to or greater than 0.345 per cent.

The average value for iron content of myoglobin, 0.323 per cent, is in good agreement with the composition calculated from the molecular weights obtained by the sedimentation-diffusion studies (see Table I) discussed above. It agrees substantially with values for iron in horse hemoglobin found by Zinoffsky (14) and Valer (13). When used to calculate the molecular weight of myoglobin, 0.323 per cent gives 17,288. This result agrees well with Polson's (6, 10) determination of the molecular weights of myoglobin, 17,200 and 17,500.

SUMMARY

A method to isolate and purify myoglobin of horse heart is described. The theoretical percentage composition with respect to iron in myoglobin is believed to be nearer 0.323 than the commonly accepted value 0.345. Eight preparations of myoglobin were analyzed. They were found to have values for iron ranging from 0.305 to 0.352 per cent, with an average of 0.323 per cent. When used to calculate the molecular weight of myoglobin, 0.323 per cent gives 17,300, which is in good agreement with the results of sedimentation and diffusion studies of myoglobin.

BIBLIOGRAPHY

1. Bergmann, M., and Niemann, C., *J. Biol. Chem.*, **118**, 301 (1937).
2. Delory, G. E., *Analyst*, **68**, 5 (1943).
3. Cohn, E. J., and Edsall, J. T., Proteins, amino acids and peptides, American Chemical Society monograph series, New York (1943).
4. Drabkin, D. L., *Am. J. Med. Sc.*, **209**, 268 (1945).
5. Millikan, G. A., *Physiol. Rev.*, **19**, 503 (1939).
6. Polson, A., *Kolloid-Z.*, **87**, 149 (1939).
7. Roche, J., and Derrien, Y., *Compt. rend. Acad. sc.*, **214**, 192 (1942).
8. Roche, J., and Vieil, H., *Compt . rend. Acad. sc.*, **210**, 314 (1940).
9. Rossi, A., and Aragona, C., *Boll. Soc. Ital. biol. sper.*, **17**, 206 (1942).
10. Svedberg, T., and Pedersen, K. O., The ultracentrifuge, Oxford (1940).
11. Theorell, A. H. T., *Biochem. Z.*, **252**, 1 (1932).
12. Theorell, H., and de Duve, C., *Arch. Biochem.*, **12**, 113 (1947).
13. Valer, J., *Biochem. Z.*, **190**, 444 (1927).
14. Zinoffsky, O., *Z. physiol. Chem.*, **10**, 16 (1886).

COMPARATIVE GROWTH ON DIETS CONTAINING TEN AND NINETEEN AMINO ACIDS, WITH FURTHER OBSERVATIONS UPON THE RÔLE OF GLUTAMIC AND ASPARTIC ACIDS*

By WILLIAM C. ROSE, M. JANE OESTERLING, and MADELYN WOMACK†

(*From the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana*)

(Received for publication, August 2, 1948)

In a series of papers extending over many years evidence has been presented regarding the nutritive rôle of each of the recognized components of proteins. The results have demonstrated that ten amino acids are essential dietary components for the rat. The exclusion from the food of any one of these, other than arginine, leads to a profound nutritive failure, loss in weight, and eventual death. Arginine differs in several respects from other constituents of proteins. First, it can be synthesized by the rat (1), but not at a rate commensurate with the needs of the organism for *maximum* growth. Thus, young animals which are deprived of arginine gain much less rapidly than do their litter mates which receive this amino acid (2). Because of this fact, arginine is classified in this laboratory as an essential component of the food. On the other hand, arginine is unique in that it can be replaced in part for growth purposes by proline or glutamic acid, though not by hydroxyproline (3). These relationships are believed to be associated with the ability or inability of the organism to effect the interconversion of the amino acids in question (*cf.* (3–6)).

In connection with the above studies two points require further consideration. The first has to do with the nutritive efficiency of diets in which the ten essential amino acids are virtually the sole sources of nitrogen. The second relates to the precise status of glutamic acid as a growth stimulant *in the presence of arginine*. No data have been presented from this laboratory regarding the comparative growth behavior of animals receiving mixtures of ten and nineteen amino acids. Several years ago, experiments of this nature were conducted under the most favorable conditions then possible. At that time, the conclusion was reached, and stated on several occasions without the submission of evidence (7, 8), that animals upon such simplified diets gain in weight as rapidly as they do when all of the com-

---

* Aided by grants from the Rockefeller Foundation, the Nutrition Foundation, Inc., and the Graduate School Research Fund of the University of Illinois.

† Present address, Bureau of Human Nutrition and Home Economics, United States Department of Agriculture, Beltsville, Maryland.

ponents of proteins are supplied preformed. We still believe that this conclusion was correct for the experimental conditions then employed. However, it is not true for the improved dietary régime now used in such studies. As pointed out previously (3), certain moderate deficiencies could not be detected by the growth method until slowly acquired information concerning the nutritive requisites of the rat, and the advent of adequate supplies of crystalline vitamins, enabled us to overhaul thoroughly our basal ration (2).

The primary objective of the present paper was to establish the efficacy, for growth purposes, of mixtures of the essential amino acids. Incidental to these studies, additional information has been obtained concerning the second question mentioned above; namely, the dietary rôle of glutamic acid. Elsewhere (3), attention has been directed to the fact that the growth status of this amino acid is somewhat uncertain. It is less than one-third as effective as arginine when added to a basal ration which is devoid of both of these compounds and the prolines. On the other hand, its addition to a diet carrying sixteen amino acids *including* arginine increases significantly the rate of gain of the experimental subjects. These rather anomalous observations appeared to necessitate additional experiments before one could classify glutamic acid with respect to its function in growth. Accordingly, a considerable number of tests have been conducted for the purpose of determining the *magnitude* of the stimulation exerted by this amino acid when incorporated in diets containing arginine. The results are summarized below.

EXPERIMENTAL

Throughout the investigation, male weanling rats served as the subjects. Each animal was housed in a separate cage, and was permitted to consume food and water *ad libitum*. All tests were continued for 28 days. The amino acids which furnished the nitrogen of the rations were purified invariably until they yielded correct analytical values.

In Table I is presented the composition of three amino acid mixtures which served as the basis of the comparative tests. Mixture XXIII contained all of the amino acids known to be present in proteins except citrulline. The absence of the latter is without effect upon the rate of gain of the rat, as has been shown by numerous experiments in this laboratory. The mixture furnishes each essential in excess quantity, without unnecessary waste, and supports as rapid growth as any preparation we have succeeded in devising. It has been used in hundreds of feeding trials in this laboratory, and is now regarded as our standard. Mixture XXIV was composed of the ten essential amino acids only. Its effective nitrogen, *i.e.* the nitrogen furnished by the *physiologically active* amino acids, was identical with that of Mixture XXIII. Mixture XXV was analogous in composition to Mixture XXIV except that it contained glutamic acid. By a proportionate adjustment downward in the amounts of the other components, its effective nitrogen was maintained at the same level as that present in Mixtures XXIII and XXIV.

TABLE I

Composition of Amino Acid Mixtures

	Mixture XXIII		Mixture XXIV		Mixture XXV	
	Physiologically active	As used	Physiologically active	As used	Physiologically active	As used
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Glycine	0.1	0.10				
Alanine	0.2	0.40*				
Serine	0.1	0.20*				
Valine	1.0	2.00*	1.32	2.64*	1.14	2.28*
Leucine	1.2	1.20	1.58	1.58	1.37	1.37
Isoleucine	0.8	1.60*	1.06	2.12*	0.91	1.82*
Cystine	0.2	0.20				
Methionine	0.8	0.80*	1.06	1.06*	0.91	0.91*
Threonine	0.7	1.40*	0.92	1.84*	0.80	1.60*
Phenylalanine	1.2	1.20*	1.58	1.58*	1.37	1.37*
Tyrosine	0.6	0.60				
Proline	0.2	0.20				
Hydroxyproline	0.1	0.10				
Tryptophan	0.4	0.40	0.53	0.53	0.46	0.46
Aspartic acid	0.2	0.40*				
Glutamic "	2.0	2.00			2.00	2.00
Lysine	1.2		1.58		1.37	
" monohydrochloride		1.50		1.98		1.71
Histidine	0.7		0.92		0.80	
" monohydrochloride monohydrate		0.95		1.24		1.08
Arginine	0.4		0.53		0.46	
" monohydrochloride		0.50		0.64		0.56
Sodium bicarbonate		1.27		1.66		1.44
	12.1	17.02	11.08	16.87	11.59	16.60

* Racemic acids.

In Table II is shown the make-up of Diets 1 to 3 inclusive, containing respectively nineteen, ten, and eleven amino acids. The unknown nitrogen present in the liver extract did not exceed 32 mg. per 100 gm. of food, and consequently could not have contributed significant amounts of glutamic acid or other amino acids. The diets were appropriately supplemented with vitamins. For this purpose, the quantities listed in Table III were thoroughly admixed with each kilo of ration.

The results of the experiments are presented in Table IV. In Series I a comparison was made of the growth of animals upon diets containing ten and nineteen amino acids. For this purpose, thirty-five male rats from five litters were divided as equitably as possible between the two diets. As will be observed, the animals which received the ten essential amino acids showed a mean gain in 28 days of 77.4 ± 1.38 gm., while the positive controls which consumed nineteen amino acids manifested a mean gain of 93.1 ± 1.95 gm. The mean difference (15.7 gm.) is highly significant, and indicates that the simpler amino acid mixture is considerably less effective than the more complex one as a source of nitrogen for growth.

TABLE II

Composition of Diets

	Diet 1	Diet 2	Diet 3
	gm.	*gm.*	*gm.*
Amino acid Mixture XXIII	17.02		
" " " XXIV		16.87	
" " " XXV			16.60
Sucrose	15.00	15.00	15.00
Dextrin	59.23	59.38	59.65
Cellu flour	2.00	2.00	2.00
Salt mixture*	4.00	4.00	4.00
Corn oil	2.00	2.00	2.00
Vitamin A and D concentrate†	0.05	0.05	0.05
Inositol	0.10	0.10	0.10
Choline chloride	0.20	0.20	0.20
Liver extract‡	0.40	0.40	0.40
	100.00	100.00	100.00

* Jones and Foster (9).

† This contained 65,000 U. S. P. units of vitamin A and 13,000 U. S. P. units of vitamin D per gm.

‡ Wilson's liver powder, 1:20.

Recently, Womack, and Rose ((3) p. 46) observed that animals upon rations containing L-tryptophan made somewhat better gains than did controls upon like diets carrying the same weight of DL-tryptophan. This finding was unexpected inasmuch as the optical isomers of this amino acid are generally regarded as equally effective in the rat for growth purposes (10, 11). The experiment summarized as Series I in the present paper antedated the observation of Womack and Rose, and involved the use of DL-tryptophan. In view of this fact, it became necessary to repeat the experiment under identical conditions except for the use of L-tryptophan. The results are summarized as Series II in Table IV. In this series three groups, totaling 101 animals from twenty-seven litters, received diets containing respectively the ten essentials, the ten essentials plus glutamic acid, and nineteen amino acids. Disregarding for the moment the second group which received eleven amino acids, one will observe that the animals upon the ten essentials showed a mean gain of 79.1 ± 0.82 gm., while the controls upon nineteen amino acids manifested a mean gain of 108.4 ± 1.28 gm. Thus, the mean difference was 29.3 gm., or almost twice as great as that in

TABLE III

Vitamin Supplements

	Added to each kilo of diet
	mg.
Thiamine hydrochloride	5
Riboflavin	10
Pyridoxine hydrochloride	5
Nicotinic acid	5
Calcium *d*-pantothenate	25
p-Aminobenzoic acid	300
α-Tocopherol	25
2-Methyl-1,4-naphthoquinone	2
	γ
Biotin	100

TABLE IV

Growth Effects of Glutamic and Aspartic Acids

The experiments covered 28 days each.

Series No.	No. of animals (males throughout)	Mean gain in weight and probable error of mean	Nature of amino acid mixture
		gm.	
I*	18	77.4 ± 1.38	10 amino acids (Mixture XXIV)
	17	93.1 ± 1.95	19 " " (" XXIII)
II	32	79.1 ± 0.82	10 " " (" XXIV)
	38	91.6 ± 1.07	11 " " (" XXV)
	31	108.4 ± 1.28	19 " " (" XXIII)
III	34	97.2 ± 0.94	18 " " (no glutamic acid)
	33	103.2 ± 1.37	19 " " (Mixture XXIII)
IV	19	109.9 ± 1.28	18 " " (no aspartic acid)
	19	111.6 ± 2.17	19 " " †

* In Series I the amino acid mixtures contained DL-tryptophan; in all other series L-tryptophan was employed.

† This mixture was identical with Mixture XXIII except that it contained 2.0 per cent of L-aspartic acid.

Series I. Evidently, a mixture of all of the components of proteins is distinctly superior in nutritive quality to one carrying the ten essentials only. The task of synthesizing ten amino acids *simultaneously* appears to present too great a burden upon the chemical resources of the cells to permit the latter to keep pace with the needs of the organism for *optimum* growth. The astounding fact to be noted is that, in the young rat, the tissues succeed in manufacturing ten of their own constituents at rates which allow bodily gains of 2.8 gm. per day.

The finding that a mixture of ten amino acids is inferior to one containing nineteen is contrary to our observation of 10 years ago with the use of a less satisfactory basal ration. Probably the divergence is attributable to defects in our earlier diets involving constituents other than the amino acids. Thus, inadequate quantities of vitamins, and perhaps of certain non-nitrogenous components of the food, may have limited growth even when all amino acids were present. This possibility is supported by the fact that the best growth then observed was much less than is now obtained regularly.

In this connection, reference must be made briefly to a paper of Albanese and Irby (12) in which the authors report that young rats lose weight upon a diet containing the essential amino acids in approximately the proportion found in casein. The paper is mentioned with reluctance inasmuch as its conclusions have already been refuted by Martin (13) and Kinsey and Grant (14). Albanese and Irby suggest that the inadequacy of their diet may have been due in part to toxic effects of the "unnatural forms of certain amino acids" used in the ration. This explanation is extremely improbable.[1] A more reasonable one is that the diet was deficient in isoleucine. The latter is said to have been supplied as a "*l*-leucine-isoleucine mixture," but no information is presented as to the amount of isoleucine which it contained. In the experiments of Martin, and of Kinsey and Grant, growth occurred invariably upon the diets containing only ten amino acids, but the mean daily gains of the rats were considerably less than those recorded in the present paper. Somewhat earlier, Bauer and Berg (15) had shown that the ten essentials suffice for the slow growth of mice.

The data summarized as Series I and II (Table IV) lend support to the idea expressed elsewhere (3) that L-tryptophan, under certain conditions, permits better growth than the same amount of DL-tryptophan. As will be observed, the two groups of animals which ingested ten amino acids made almost identical mean gains. On the other hand, with the diet containing nineteen amino acids, the animals which received L-tryptophan showed a mean gain which was 15.3 gm. in excess of that achieved by the subjects which consumed the racemic compound. Thus, the superiority of L-tryptophan is evident only with the ration which otherwise possessed the greater nutritive quality. Statistically the difference is highly significant. Perhaps one may assume from the findings that the inversion of D-tryptophan keeps pace with the needs of the organism for *limited* growth, such as that which occurs when all of the non-essential amino acids must be synthesized concurrently, but is not sufficiently rapid to meet the requirements for *maximum* growth. Possible objections may be raised to this interpretation; but if it should prove to be correct, the behavior of DL-tryptophan will provide another illustration of how the *rate* of a reaction may affect the magnitude of the gain. The problem is being investigated further in this laboratory at the present time.

---

[1] A personal communication from Dr. K. A. J. Wretlind of the Karolinska Institutet, Stockholm, announces that he has obtained weight gains in rats of 0.3 to 0.9 gm. daily upon diets containing 10 to 20 per cent of a mixture of *only* the racemic forms of the ten essential amino acids. His paper is now in press.

The data in Table IV are illuminating also with respect to the growth effects of glutamic acid. In Series II, the inclusion of this amino acid in the food increased the mean gain of the subjects from 79.1 ± 0.82 to 91.6 ± 1.07 gm. Thus, the mean difference was 12.5 gm. However, despite the stimulatory action of glutamic acid, the accomplishment of the animals upon eleven amino acids was quite inferior to that of the controls which received nineteen amino acids. The respective gains were 91.6 ± 1.07 and 108.4 ± 1.28 gm., with a mean difference of 16.8 gm. Both increments are highly significant statistically.

Taken by themselves, the findings in Series II are difficult to interpret. Do they imply that glutamic acid has a specific rôle in growth, or do they merely reflect the decreasing synthetic burden upon the animals as the number of preformed amino acids in the food is increased? In order to throw further light upon this question, a comparison was made of the growth of animals upon two diets which were more complete with respect to their amino acid content, and were comparable in composition except that one contained 2 per cent of L-glutamic acid and the other was devoid of this substance. Thus, the general procedure in this experiment was analogous to that followed in most of our previous investigations involving the nutritive rôle of the amino acids. For the purpose in hand, 67 male rats from nineteen litters were divided as equitably as possible into two groups. The positive controls received a ration containing Mixture XXIII. The diet of their litter mates carried a similar mixture from which glutamic acid alone had been excluded. The effective nitrogen content of the two diets was equalized by an appropriate and proportional increase in each of the eighteen amino acids in the second mixture. Thus, the only variable was the glutamic acid content of the food.

The results of the tests are summarized as Series III in Table IV. The data show that the mean gains of the rats which respectively were deprived of and received glutamic acid were 97.2 ± 0.94 and 103.2 ± 1.37 gm.; hence, the mean difference amounted to 6.0 gm. in favor of the animals which consumed the more complete ration. A difference of this magnitude is of doubtful significance. The ratio of the mean difference to the probable error of the difference is 3.6. This implies that the odds against the difference being due to chance alone are approximately 65 to 1. Most investigators would not be inclined to regard this as convincing. In any event, the exclusion of glutamic acid from a ration containing all of the amino acids except citrulline does not exert the inhibition in growth one would expect of an indispensable dietary component. Under like conditions, the absence of arginine, the least effective of the essentials, induces a much more profound influence upon the rate of gain in body weight. It seems necessary, therefore, in accordance with our recent tentative suggestion (3), to classify glutamic acid as a dispensable amino acid for the rat despite the fact that it, like proline, can replace in part the arginine requirement of this species.

TABLE V

Classification of Amino Acids with Respect to Their Growth Effects in the Rat

Essential	Non-essential
Lysine	Glycine
Tryptophan	Alanine
Histidine	Serine
Phenylalanine	Cystine*
Leucine	Tyrosine†
Isoleucine	Aspartic acid
Threonine	Glutamic " ‡
Methionine	Proline‡
Valine	Hydroxyproline
Arginine§	Citrulline

* Cystine can replace about one-sixth of the methionine requirement, but has no growth effect in the *absence* of methionine.

† Tyrosine can replace about one-half of the phenylalanine requirement, but has no growth effect in the *absence* of phenylalanine.

‡ Glutamic acid and proline can serve individually as rather ineffective substitutes for arginine in the diet. This property is not shared by hydroxyproline.

§ Arginine can be synthesized by the rat, but not at a sufficiently rapid rate to meet the demands of *maximum* growth. Its classification, therefore, as essential or non-essential is purely a matter of definition.

Series IV of Table IV summarizes, for comparative purposes, the results of similar tests upon the growth effects of aspartic acid. Thirty-eight young rats from eleven litters were divided into two groups of nineteen animals each, and were placed upon comparable diets except that one contained 2 per cent of aspartic acid and the other was devoid of this amino acid. The nitrogen content of the two rations was equalized as described for the tests of Series III. The data demonstrate clearly that the exclusion of aspartic acid is without effect upon the rate of gain. This is in agreement with the conclusion of Rose and Fierke (16) in earlier experiments involving the use of a much less satisfactory basal ration.

Inasmuch as the present paper is the last from this laboratory dealing specifically with the growth effects of the amino acids in the rat, their final classification into essential and non-essential components of the food is summarized in Table V. This table is a modification of one published elsewhere (17) more than 10 years ago. Since that time, certain interrelationships have been discovered which were not then appreciated. Furthermore, during the same interval marked species differences have come to light. No longer is one warranted in referring to amino acids as dispensable or indispensable without designating the species in which the tests were made, and indicating the criterion used as the basis of the classification. The conclusions presented in Table V apply to the growth of the rat only.

SUMMARY

Experiments have been conducted upon a relatively large number of young rats to determine the comparative efficiency for growth purposes of mixtures of ten and nineteen amino acids. Contrary to our observations of more than a decade ago, involving the use of a less satisfactory basal ration, the results demonstrate that the simpler mixture possesses a lower nutritive value as measured by the relative gains in weight of the subjects. During periods of 28 days, animals which receive the ten essentials only, in an otherwise adequate diet, gain approximately 70 to 75 per cent as much as their litter mates which consume nineteen amino acids.

Further experiments upon the rôle of glutamic acid in growth indicate that the addition of this amino acid to a ration containing the ten essentials exerts a significant stimulatory effect upon the rate of gain, but that its removal from a diet containing nineteen amino acids is followed by a very slight inhibition which statistically is of doubtful significance. Under the latter conditions, its influence, if any, upon growth is certainly much less than that manifested by arginine. In view of these facts, glutamic acid is classified as a dispensable dietary component for the rat.

In confirmation of an earlier investigation from this laboratory, the exclusion of aspartic acid from the food does not affect the growth of the rat.

A final classification of the amino acids with respect to their rôle in the growth of the rat is presented.

BIBLIOGRAPHY

1. Scull, C. W., and Rose, W. C., *J. Biol. Chem.*, **89,** 109 (1930).
2. Borman, A., Wood, T. R., Black, H. C., Anderson, E. G., Oesterling, M. J., Womack, M., and Rose, W. C., *J. Biol. Chem.*, **166,** 585 (1946).
3. Womack, M., and Rose, W. C., *J. Biol. Chem.*, **171,** 37 (1947).
4. Clutton, R. F., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **132,** 227 (1940).
5. Roloff, M., Ratner, S., and Schoenheimer, R., *J. Biol. Chem.*, **136,** 561 (1940).
6. Stetten, M. R., and Schoenheimer, R., *J. Biol. Chem.*, **153,** 113 (1944).
7. Rose, W. C., *Physiol. Rev.*, **18,** 109 (1938).
8. Rose, W. C., *Proc. Inst. Med. Chicago*, **12,** 98 (1938).
9. Jones, J. H., and Foster, C., *J. Nutr.*, **24,** 245 (1942).
10. du Vigneaud, V., Sealock, R. R., and Van Etten, C., *J. Biol. Chem.*, **98,** 565 (1932).
11. Berg, C. P., *J. Biol. Chem.*, **104,** 373 (1934).
12. Albanese, A. A., and Irby, V., *Science*, **98,** 286 (1943).
13. Martin, G. J., *Proc. Soc. Exp. Biol. and Med.*, **55,** 182 (1944).
14. Kinsey, V. E., and Grant, W. M., *Science*, **99,** 303 (1944).
15. Bauer, C. D., and Berg, C. P., *J. Nutr.*, **26,** 51 (1943).
16. Rose, W. C., and Fierke, S. S., *J. Biol. Chem.*, **143,** 115 (1942).
17. Rose, W. C., *Science*, **86,** 298 (1937).

ENZYMATIC DEHALOGENATION OF CERTAIN BROMINATED AND CHLORINATED COMPOUNDS

By LEON A. HEPPEL and VIRGINIA T. PORTERFIELD*

(*From the Laboratory of Physical Biology, National Institute of Health, Bethesda, Maryland*)

(Received for publication, July 22, 1948)

The dehalogenation *in vivo* of brominated aliphatic hydrocarbons such as methyl bromide (1) and bromochloromethane (2) has been demonstrated by recovery of inorganic bromide from blood and urine. The intracellular formation of bromide and methyl alcohol from methyl bromide has been advanced as an explanation for its prolonged toxic effect (3). Evidence for the splitting of chlorinated aliphatic hydrocarbons has not been obtained. Thus, methyl alcohol could not be detected in the blood of animals exposed to methyl chloride (4). In the present investigation it was found that protein fractions from liver catalyzed the hydrolysis of bromochloromethane (CH_2BrCl), dibromomethane, and dichloromethane to hydrogen ion, halide ion, and formaldehyde. The enzyme system was activated by cyanide and sulfhydryl compounds.

Methods

Preparation of Tissues—Slices of tissue approximately 0.3 mm. thick were cut free-hand and immersed in Krebs-Ringer-phosphate solution (5). Homogenates were prepared according to Potter and Elvehjem (6). Extracts were made by centrifuging homogenates for 10 minutes at 11,000 R.P.M. in an angle centrifuge at 2°. The solution used for preparing homogenates and extracts was similar to that of Buchanan, Hastings, and Nesbett (7).

Measurement of Enzymatic Dehalogenation—Inorganic bromide formed in an hour from brominated hydrocarbons was proportional to the concentration of tissue. The reaction was stopped by addition of trichloroacetic acid (final concentration 5 per cent). Bromide was determined by Friedman's procedure (8) after removal of organic brominated compounds by aeration for 15 minutes at 40°.

Assay for enzymatic activity was also carried out by measuring the rate of CO_2 evolution from a bicarbonate buffer due to the liberation of hydrogen ion from halogenated compounds. Warburg flasks were gassed in pairs for 8 minutes, at a rate of 100 ml. per minute, with 95 per cent N_2-5 per cent CO_2. The gas stream was first bubbled through 500 ml. of fluid containing the desired concentration of halogenated compound. By this procedure the concentration of volatile substrate in the reaction vessels was 80 to 95 per cent of that in the reservoir wash bottle (9). After a 5 minute equilibration period CO_2 evolution was measured for 20 minutes, during which time the rate was linear.

---

* Part of the material in this paper was taken from a thesis submitted by Virginia T. Porterfield to the Chemistry Department of the Graduate School of Georgetown University in partial fulfilment of the requirement for the degree of Master of Science, June, 1948.

Determination of Formaldehyde—The incubation mixture was distilled with acid and the chromotropic acid procedure for formaldehyde was applied to the distillate (10). Fraenkel-Conrat *et al.* (11) have pointed out that proteins rich in indole groups will bind formaldehyde irreversibly even under conditions of combined acid hydrolysis and distillation. Table I indicates that formaldehyde could be recovered in satisfactory amounts when added to liver extracts, provided that a suitable choice of acid and method of distillation was made. It should be noted that the commonly used procedure of distillation with sulfuric acid gave low recoveries.

Table I

Per Cent Recovery of Formaldehyde Added to Rat Liver Extracts

Formaldehyde, usually 120 γ, was added to 2.5 ml. of liver extract, equivalent to 375 mg. of liver. The volume was made to 10 ml., after which the containers were stoppered and kept at 25° for 15 minutes. Then acid was added and the volume was made up to 20 ml. Distillation was carried nearly to dryness. With steam distillation 25 ml. of distillate were collected.

	H_2SO_4		HCl		CCl_3COOH	
	20 per cent (volume)	10 per cent (volume)	20 per cent (volume)	10 per cent (volume)	20 per cent (weight)	10 per cent (weight)
Distillation	51.2 (5)*	87.1 (2)	82.7 (3)	85.3 (3)	96.0 (7)	92.1 (4)
Steam distillation	82.3 (5)	97.4 (3)	92.9 (3)	90.3 (3)	93.4 (3)	90.1 (4)

* The figures in parentheses indicate the number of determinations whose result are averaged.

Preparations Used—The CH_2BrCl[1] was a fraction distilling in a 4 foot column at 67–68°, with an index of refraction n_D^{26} of 1.4796 and a density at 25° of 1.930. The compounds CH_2Br_2, CH_2Cl_2, $CHBr_3$, $CH_2Cl\cdot CH_2Cl$, and $CHCl_3$ were also redistilled. The other compounds were Eastman Kodak products used without purification.

---

[1] This compound was obtained from the Michigan Chemical Corporation, St. Louis, Michigan. The commercial sample had a distillation range of 64.8–67.5°.

Results

Dehalogenation of Aliphatic Halogenated Hydrocarbons by Tissue Preparations—Table II shows that slices of rat liver, kidney, and spleen catalyzed the formation of bromide from CH_2BrCl. Certain other tissues were relatively ineffective under these conditions. Homogenates of liver were active but those of kidney showed greatly reduced or no activity. The cleavage of CH_2BrCl by rat liver homogenate was 3 times as rapid in nitrogen as in air. It was completely inhibited by 10^{-3} M $HgCl_2$ and by 5 minutes incubation at 60°. 50 per cent inhibition was caused by 10^{-2} M NaF and 30 per cent inhibition by 10^{-4} M $HgCl_2$.

TABLE II

Formation of Inorganic Bromide from CH_2BrCl by Rat Slices from Various Organs

Slices weighing 500 mg. and about 0.3 mm. in thickness were incubated with shaking at 37°. Incubation time, 1 hour; gas phase, air; immersion fluid, 10 ml. of Krebs-Ringer-phosphate, pH 7.4; concentration of CH_2BrCl in liquid phase, 0.011 M. The results are averages of at least two separate assays.

Organ	Inorganic bromide per gm. wet tissue per hr.
	mg.
Kidney	1.09
Liver	0.71
Spleen	0.26
Brain	0.08
Heart	0.06
Diaphragm	0.06

Table III indicates the relative amounts of bromide and formaldehyde resulting from incubation of CH_2BrCl and CH_2Br_2 with rat liver extract. The results are consistent with the following equations:

$$CH_2BrCl + H_2O = 2H^+ + Br^- + Cl^- + HCHO$$
$$CH_2Br_2 + H_2O = 2H^+ + 2Br^- + HCHO$$

In the case of CH_2Cl_2 no exact comparisons were made but the accumulation of formaldehyde was of the same order of magnitude as hydrogen ion production measured manometrically. In several experiments with CH_2BrCl formaldehyde was determined colorimetrically and also gravimetrically as the dimedon derivative (12). Satisfactory agreement was obtained and the crystalline dimedon compound had the same melting point and mixed melting point as an authentic sample.

The rate of CO_2 evolution from a bicarbonate buffer due to fixed acid production from CH_2BrCl was proportional to the concentration of tissue. This is shown in Fig. 1. The ordinates of Fig. 1 represent uncorrected values for CO_2 because volatile substrate was disappearing at a rate proportional to CO_2 formation. The true values are somewhat larger than those indicated.

Fig. 2 shows that with optimal substrate concentrations CH_2BrCl was dehalogenated about 4 times as rapidly as CH_2Br_2 or CH_2Cl_2. Chloroform was acted upon slowly and bromoform was not attacked. Several ethane derivatives were tested either manometrically or by measuring inorganic bromide. With C_2H_5Br, $CH_2Br \cdot CH_2Br$, $CH_2Cl \cdot CH_2Br$, and $CH_2Cl \cdot CH_2Cl$ the rate was somewhat slower than that observed with CH_2Br_2.

Table III

Comparison of Amounts of Bromide and Formaldehyde Formed from CH_2BrCl and CH_2Br_2 during Incubation with Rat Liver Extract

Anaerobic incubation was carried out at 37°, for 60 minutes with CH_2BrCl and 90 minutes with CH_2Br_2. Each flask contained 10 ml. of fluid, including liver extract equivalent to 375 mg. of fresh liver. The medium contained K^+, 0.13 M; Mg^{++}, 0.01 M; Cl^-, 0.15 M; Na^+, 0.03 M; phosphate, pH 7.4, 0.02 M. The concentration of substrate was 0.022 M.

	Experiment No.	Bromide production	Formaldehyde production	$\frac{Br^-}{HCHO}$
		micromoles	*micromoles*	
CH_2BrCl	1	5.4	5.2	1.0
	2	6.5	6.4	1.0
	3	9.3	8.6	1.1
Average......				1.05
CH_2Br_2	1	7.1	2.8	2.5
	2	6.5	5.3	1.2
	3	4.0	1.9	2.1
	4	3.7	1.7	2.2
Average......				2.0

Activation by Cyanide and Sulfhydryl Compounds—Crude liver extracts aged at 0°, dialyzed extracts, and ammonium sulfate fractions required activation by cyanide and either glutathione or cysteine. Table IV indicates that with the concentrations tested both glutathione and cyanide were needed for the greatest effect. Irving, Fruton, and Bergmann (13) have recorded some interesting experiments with papain and cathepsin, whose requirements for activation are somewhat similar to those recorded here.

Purification of Enzyme Activity—A 5-fold purification of the crude rat liver extracts by ammonium sulfate fractionation could be demonstrated. A fraction was precipitated between 0.45 and 0.55 saturation. This fraction had a pH optimum in the range 7.1 to 7.4. At pH 5.1 and 8.5 enzymatic activity was lost after several hours at 2°. At pH 7.4 and 2° the activity decreased by one-third over a period of several weeks.

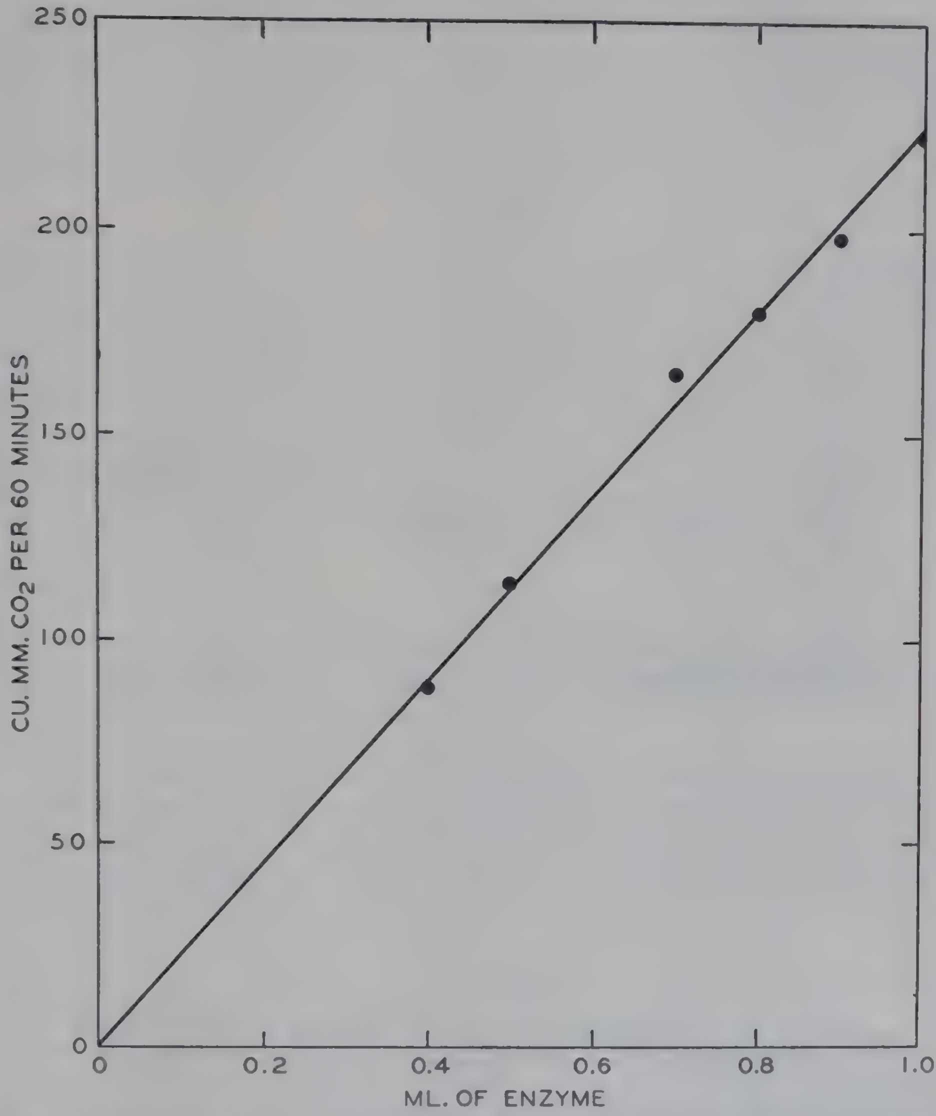


Fig. 1. Relationship between reaction velocity and concentration of enzyme. An ammonium sulfate fraction of rat liver with 10 mg. of protein per ml. was used. The Warburg flasks also contained K^+, 0.05 M; Cl^-, 0.048 M; Na^+, 0.017 M; Mg^{++}, 0.004 M; CN^-, 0.01 M; HCO_3^-, 0.017 M; glutathione, 1.7×10^{-3} M; CH_2BrCl, 0.022 M; total volume, 3 ml.

Effect of Exposure of Rats to CH_2BrCl on Enzymatic Activity of Liver—Adult male rats were exposed 6 to 7 hours daily, 5 days a week, to an atmosphere containing 1,000 parts per million of CH_2BrCl. After twenty to thirty exposures the rats were killed and homogenates of their livers were incubated in air with CH_2BrCl. Seventeen of these preparations formed an

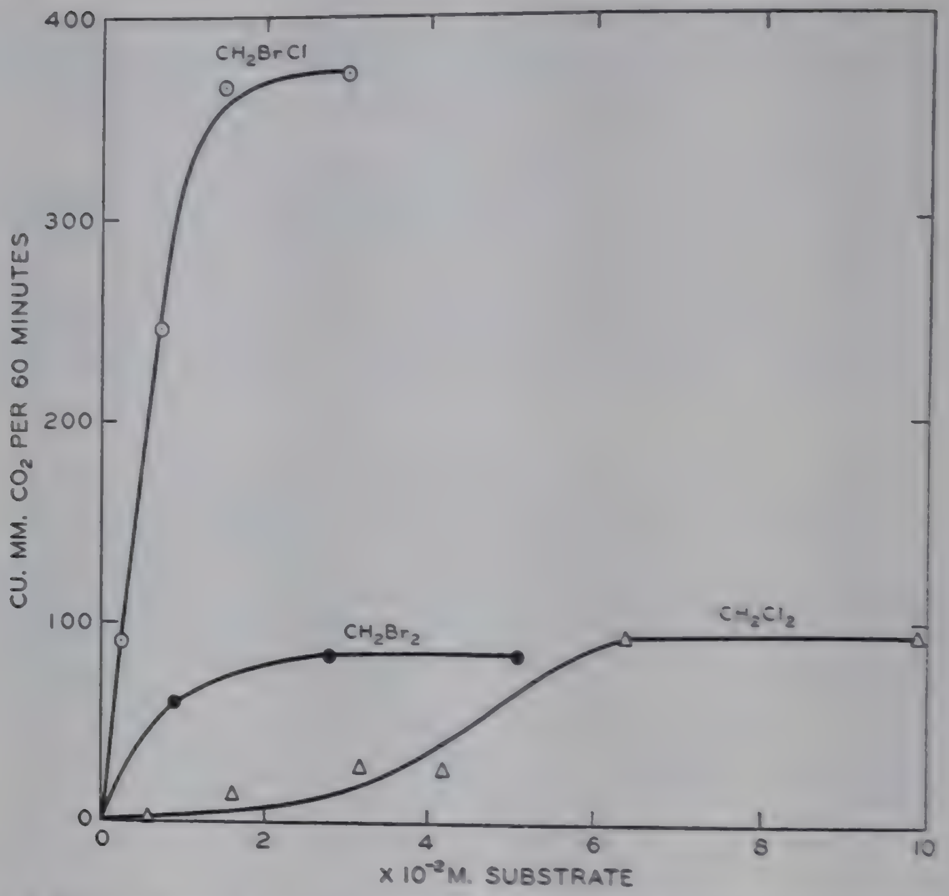


FIG. 2. Comparison of rates of enzymatic hydrolysis of different substrates. Each flask contained 1 ml. of crude rat liver extract with a protein content of 60 mg. The flasks also contained K^+, 0.05 M; Cl^-, 0.048 M; Na^+, 0.017 M; Mg^{++}, 0.004 M; CN^-, 0.01 M; HCO_3^-, 0.017 M; glutathione, 1.7×10^{-3} M; CH_2BrCl, 0.022 M; total fluid volume, 3 ml.

TABLE IV

Requirements of Ammonium Sulfate Fraction for Glutathione and Cyanide in Order to Catalyze Hydrolysis of CH_2BrCl

The Warburg flasks contained a fluid volume of 3 ml.; K^+, 0.04 M; Cl^-, 0.048 M; Na^+, 0.017 M; Mg^{++}, 0.004 M; HCO_3^-, 0.017 M; CH_2BrCl, 0.022 M; enzyme solution, 1 ml., with 9 mg. of protein.

Concentration of GSH	Concentration of CN^-	Enzyme activity
M	*M*	*c.mm. CO_2 per hr.*
0	0.01	0
2.1×10^{-4}	0.01	74
2.1×10^{-3}	0.01	195
2.1×10^{-4}	0	0
2.1×10^{-3}	0	46
1.8×10^{-2}	0	26

verage of 1.32 mg. of bromide per gm. of wet tissue per hour compared with a figure of 0.81 for homogenates from fourteen unexposed control rats. The difference was statistically highly significant ($P < 0.001$ with Fisher's method (14)).

Formaldehyde was demonstrated in the livers of rats killed after an hour of deep narcosis with CH_2BrCl. The concentration was only 1.1 mg. per 100 gm. of liver. This may be due to oxidation *in vivo* of most of the formaldehyde resulting from hydrolysis of CH_2BrCl.

SUMMARY

1. The enzymatic dehalogenation of CH_2BrCl, CH_2Br_2, CH_2Cl_2, $CH_2Br\cdot CH_2Br$, $CH_2Cl\cdot CH_2Cl$, $CHCl_3$, C_2H_5Br, and $CH_2Cl\cdot CH_2Br$ has been demonstrated in liver extracts. The products of reaction with CH_2BrCl, CH_2Br_2, and CH_2Cl_2 were formaldehyde, halide ion, and hydrogen ion.
2. The enzyme system required activation by cyanide and either glutathione or cysteine.
3. A 5-fold purification was achieved by fractionation of liver extracts with ammonium sulfate.

The authors are indebted to Dr. Arthur Kornberg for helpful advice and criticism. The technical assistance of Mrs. Evelyn G. Peake is gratefully acknowledged.

BIBLIOGRAPHY

1. Irish, D. D., Adams, E. M., Spencer, H. C., and Rowe, V. K., *J. Ind. Hyg. and Toxicol.*, **23**, 408 (1941).
2. Svirbely, J. L., Highman, B., Alford, W. C., and von Oettingen, W. F., *J. Ind. Hyg. and Toxicol.*, **29**, 382 (1947).
3. Miller, D. P., and Haggard, H. W., *J. Ind. Hyg. and Toxicol.*, **25**, 423 (1943).
4. Smith, W. W., *J. Ind. Hyg. and Toxicol.*, **29**, 185 (1947).
5. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis (1945).
6. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
7. Buchanan, J. M., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, **145**, 715 (1942).
8. Friedman, M. M., *J. Biol. Chem.*, **144**, 519 (1942).
9. Jowett, M., and Quastel, J. H., *Biochem. J.*, **31**, 1101 (1937).
10. MacFadyen, D. A., *J. Biol. Chem.*, **158**, 107 (1945).
11. Fraenkel-Conrat, H., Brandon, B. A., and Olcott, H. S., *J. Biol. Chem.*, **168**, 99 (1947).
12. Yoe, J. H., and Reid, L. C., *Ind. and Eng. Chem., Anal. Ed.*, **13**, 238 (1941).
13. Irving, G. W., Jr., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **139**, 569 (1941).
14. Fisher, R. A., Statistical methods for research workers, London, 10th edition, (1946).

HEPATORENAL FACTORS IN CIRCULATORY HOMEOSTASIS

IX. THE IDENTIFICATION OF THE HEPATIC VASODEPRESSOR SUBSTANCE, VDM, WITH FERRITIN*

By ABRAHAM MAZUR and EPHRAIM SHORR

WITH THE TECHNICAL ASSISTANCE OF IRENE LITT

(*From the Department of Medicine, Cornell University Medical College, and The New York Hospital, New York*)

(Received for publication, June 26, 1948)

Earlier studies from this laboratory (1) have revealed the regular participation in experimental shock of two hitherto undescribed vasotropic principles, a vasoexcitor material (VEM) of renal and a vasodepressor material (VDM) of hepatic origin. The renal vasoexcitor appeared during the initial compensatory or hyperreactive phase of shock, the hepatic vasodepressor during the subsequent decompensatory or hyporeactive phase. The effect of the renal vasoexcitor on the terminal vascular bed of an animal in shock was to increase spontaneous vasomotion and enhance the reactivity of the terminal arterioles and precapillary sphincters to the topical application of epinephrine. The hepatic vasodepressor, on the other hand, brought about a reduction of vasomotion and a depression of the reactivity of these terminal muscular vessels to epinephrine. These principles were detected in both blood and tissue extracts by the vascular effects which they induced when injected intravenously into anesthetized normal rats whose mesoappendix was exposed for direct visualization. Enhancement or depression of the reactivity of the blood vessels to the topical application of epinephrine permitted the differentiation between the vasoexcitor and vasodepressor principles (2).

The present study is concerned with the purification and chemical characterization of VDM of hepatic origin and with its relationship to the VDM present in the blood during the hyporeactive phase of shock. Although our studies have shown that a vasodepressor material of comparable activity is also formed by skeletal muscle and spleen during the shock syndrome, attention in this study has been largely focused on the VDM of hepatic origin.

With the progressive concentration of VDM activity in extracts of beef,

---

* Aided by grants from the Josiah Macy, Jr., Foundation, Eli Lilly and Company, the Postley Hypertension Fund, and the United States Public Health Service.

We wish to acknowledge our indebtedness to Dr. G. H. A. Clowes and Dr. E. D. Campbell of the Lilly Research Laboratories for the many ways in which they have assisted in these studies.

dog, rat, and human liver, preparations were obtained which were increasingly concentrated with respect to the ratio of iron to nitrogen. The iron component had the characteristics of colloidal ferric hydroxide. The possibility was therefore explored that hepatic VDM might be identical with ferritin, an unusual iron-protein first crystallized by Laufberger (3) and shown to be present in both liver and spleen. This identification was established by a combination of chemical and immunochemical procedures. It was further found that ferritin (or apoferritin) was identical with naturally occurring VDM, present in the liver and blood during hyporeactive shock and in the blood during the chronic stage of experimental renal and human essential hypertension.

EXPERIMENTAL

Our previous studies (1) have established the conditions *in vivo* and *in vitro* for the formation and inactivation of VDM. These have been listed in Table I. This information made it possible to set up appropriate conditions not only for obtaining maximal yields of hepatic VDM for chemical fractionation, but also for clarifying, by immunochemical procedures, the relationship of the purified VDM of hepatic origin to naturally occurring VDM.

The method of preparation of active VDM solutions for purposes of chemical fractionation was based on Reaction 4, Table I. The rat mesoappendix technique was utilized for the determination of the potency of VDM during the process of fractionation (2). The test sample was diluted with 0.9 per cent saline until the intravenous injection into a test rat of 0.5 ml. resulted in an inhibition of the epinephrine response which lasted from 20 to 40 minutes. This depression of epinephrine reactivity is similar to that induced by 0.5 ml. of dog plasma removed during the irreversible phase of hemorrhagic shock. These bioassays were conducted under the supervision of our associate, Dr. B. W. Zweifach.

Concentration of Beef Liver VDM—Large scale preparation of VDM was carried out with beef liver in the laboratories of Eli Lilly and Company, Indianapolis, under the direction of Dr. E. D. Campbell. The liver, collected immediately after slaughter of the animal, was packed in insulated cans and maintained at approximately body temperature for 2 hours. This procedure was effective in maintaining anaerobic conditions for the greatest proportion of the tissue. The liver was then sliced with an electric slicing machine as thin as feasible (about 2 mm.). The slices were washed by gentle mixing with 5 volumes of 0.9 per cent saline and the extract clarified in a Sharples centrifuge. This extract corresponds to Fraction A in Table II. Table II gives the method used for the concentration of VDM. The procedure was carried through Step 3 by Dr. Campbell, and the dialyzed solution, which usually represented a concentrate from 60 kilos of liver, was lyophilized and sent to our laboratory for further fractionation. Steps 4 and 6 lowered the total recoverable activity but were nevertheless advantageous, since they removed appreciable amounts of inactive material. The degree of concentration of VDM activity, by the method outlined in Table II, varied considerably from one preparation to another (see Table III).

TABLE I

Factors Governing Origin and Inactivation of VDM

For studies *in vitro* liver slices (dog, rabbit, rat) were incubated with 5 volumes of Ringer-phosphate, pH 7.4, at 37.5° for 2 hours. The centrifuged clear solution was injected into the rat to test for VDM activity. A neutral test signified absence of any effect on the reactivity of the blood vessels of the mesoappendix to topical epinephrine. Anaerobic liver slices were prepared by incubation of normal liver slices in Ringer-phosphate in N_2 for 2 hours. The slices were removed from the solution, washed with cold saline to remove any adhering VDM, and then used for further incubation.

	Experimental conditions	Rat assay
In vivo	Reaction 1. Plasma, irreversible shock (hyporeactive stage)	VDM
	" 2. Liver, irreversible shock (hyporeactive stage)	"
In vitro	" 3. Normal liver slices in O_2	Neutral
	" 4. " " " " N_2	VDM
	" 5. Anaerobic liver slices in O_2	"
Inactivation (*in vitro*)	" 6. Normal liver slices + VDM in O_2	Neutral
	" 7. " " " + " " N_2	VDM
	" 8. Anaerobic liver slices + VDM in O_2	"
	" 9. Liver slices, irreversible shock, + VDM in O_2	"

The properties of Fraction B, Table II, were those of proteins. 50 per cent of the total nitrogen of an acid hydrolysis mixture was accounted for by humin, ammonia, glutamic acid, phenylalanine, tyrosine, arginine, and histidine (4). The concentrated beef liver VDM gave negative Molisch and pentose tests and contained 0.1 per cent phosphorus. The straw-yellow VDM solutions contained protein-bound iron in variable concentrations. Addition of $Na_2S_2O_4$ and pyridine yielded no absorption bands of hemochromogens. The iron was therefore not present as a porphyrin complex.

An electrophoretic study[1] of one beef liver VDM preparation (Fraction

---

[1] We wish to express our appreciation to Dr. Kurt Stern and Mr. Jack Wagman of the Brooklyn Polytechnic Institute for the electrophoresis and ultracentrifuge determinations reported in this paper.

B) revealed a mixture of at least two protein components with diffe mobilities. Another such fraction was run in the ultracentrifuge[1] at 34 R.P.M. for 30 minutes. The original solution, before centrifugation, c tained 0.01 mg. of iron per mg. of nitrogen. After centrifugation, mos the color had concentrated in the form of a dark brown pellet at the bot of the tube. It was separated and redissolved in phosphate buffer, 7.4. This solution was found to contain 0.17 mg. of iron per mg. of ni gen and contained the VDM activity. The almost colorless supernata was very low in iron and was devoid of VDM activity.

Concentration of Dog Liver VDM—Dog liver VDM was prepared in laboratory from two sources: from slices of liver obtained from dogs hyperreactive shock and washed with saline, and from normal dog li

TABLE II

Concentration of Liver VDM Activity

The steps outlined are for the preparation of VDM from beef liver. Steps 4 6 were omitted for the preparation of VDM from dog, horse, human, and rat.

Step 1. Extract anaerobic liver slices with 3 volumes saline; centrifuge (Fraction
" 2. Adjust filtrate to pH 3.8 with 1 N HCl; heat in water bath to 80-85°; fil discard ppt.
Step 3. Concentrate filtrate in vacuo approximately 20-fold; dialyze against run tap water
Step 4. Saturate with NaCl; centrifuge ppt. and redissolve in water; dialyze
" 5. Make 30% saturated with $(NH_4)_2SO_4$; centrifuge ppt. and redissolve water; repeat 4 times; dialyze
Step 6. Saturate with NaCl; centrifuge ppt.; redissolve and repeat; dialyze
" 7. Make 30% saturated with $(NH_4)_2SO_4$; centrifuge ppt. and repeat; dialy (Fraction B

---

slices incubated in nitrogen for 2 hours at 37.5° (Reactions 2 and 4, Ta I). Maximal concentration of VDM activity was obtained by using t method outlined in Table II, except for the omission of Steps 4 and Repeated fractionation with varying concentrations of $(NH_4)_2SO_4$ yielde fraction with 1.12 mg. of iron per mg. of nitrogen, which gave a posi VDM test in concentrations of 0.0005 γ of nitrogen per 0.5 ml. of soluti In this fraction as well as in those obtained from beef liver, the iron firmly bound to the protein, from which it was not removed by dialysis the presence of 0.1 M phosphate buffer, pH 7.4, 0.05 M NaCN, 1 M acet buffer, pH 4.8, or 0.01 N HCl. The iron could be removed from the pro by dialysis in acetate buffer, pH 4.8, in the presence of $Na_2S_2O_4$. Un these conditions the addition of α,α'-dipyridyl gave rise to the pink co of the ferrous-dipyridyl complex.

Table III presents the results of a comparison of the VDM activity w ron and nitrogen content of a number of VDM preparations from beef, horse, rat, and human liver. There was no relation between VDM ity and the N content. However, within the experimental error of rat assay method, a good correlation was found between the iron con- and VDM activity.

lation of VDM to Ferritin—The presence of iron in all fractions with M activity as well as the chemical nature of the nonspecific linkage to a consideration of the possible identity of VDM with ferritin. The ploration of this possibility was facilitated by the fundamental studies Granick (5) on crystalline ferritin.

TABLE III

Correlation of VDM Activity and Fe Content

of liver	Preparation No.	Fe Content	VDM activity in terms of	
			Nitrogen	Iron
		mg. Fe per mg. N	γ	γ
Beef	1072-3	0.004	0.05	0.0002
"	1070	0.009	0.02	0.0002
"	1507	0.011	0.03	0.0003
"	1050	0.016	0.03	0.0005
"	1085	0.016	0.05	0.0008
"	1064	0.068	0.004	0.0003
"	EG*	0.604	0.001	0.0006
Human	1	0.24	0.001	0.0002
Rat	1	0.38	0.001	0.0004
"	2	0.53	0.001	0.0005
Horse	1	0.78	0.001	0.0008
Dog	2	0.311	0.001	0.0003
"	1	0.354	0.001	0.0004
"	10	0.500	0.0006	0.0003
"	3*	1.120	0.0004	0.0004

* These preparations were obtained by repeated fractionation of Fraction B, ble II, with concentrations of $(NH_4)_2SO_4$ varying from 25 to 50 per cent of satu- ion.

The addition to preparations of dog liver VDM (such as Preparation 3, ble III) of a 20 per cent solution of $CdSO_4$ to a final concentration of 2 cent resulted in the deposition of dark brown crystals typical of ferritin g. 1). The presence of ferritin in VDM preparations being established, was then necessary to determine whether ferritin was the principle ponsible for the vasotropic activity of these preparations.

To permit a comparison of the chemical and vasotropic properties of DM concentrates with ferritin, crystalline ferritin was prepared from horse spleen and liver, dog liver, and human liver obtained at autopsy. The method was essentially that of Granick (6) with some modifications.[2] Prior to the precipitation of crude ferritin by 50 per cent saturation with $(NH_4)_2SO_4$, as outlined by Granick, the solution was adjusted to pH 4.6 with 50 per cent acetic acid and allowed to stand for several hours at room temperature or overnight in the refrigerator. The precipitate which formed was centrifuged and discarded. The ferritin was then crystallized with $CdSO_4$, redissolved in 2 per cent $(NH_4)_2SO_4$, and recrystallized four times. It was next dissolved in 2 per cent $(NH_4)_2SO_4$ and dialyzed against running tap water overnight to remove most of the cadmium. The clear solution was then treated with an equal volume of saturated $(NH_4)_2SO_4$ and the precipitate recovered by centrifugation. It was redissolved in water and the reprecipitation with $(NH_4)_2SO_4$ repeated three times. This procedure served to reduce further the Cd content of the final ferritin solution. The contaminating $(NH_4)_2SO_4$ was removed by exhaustive dialysis and the concentrated ferritin solutions stored in the refrigerator with toluene as the preservative. It was found that freezing of such solutions should be avoided, since some insoluble ferritin appeared on thawing, which did not redissolve completely.

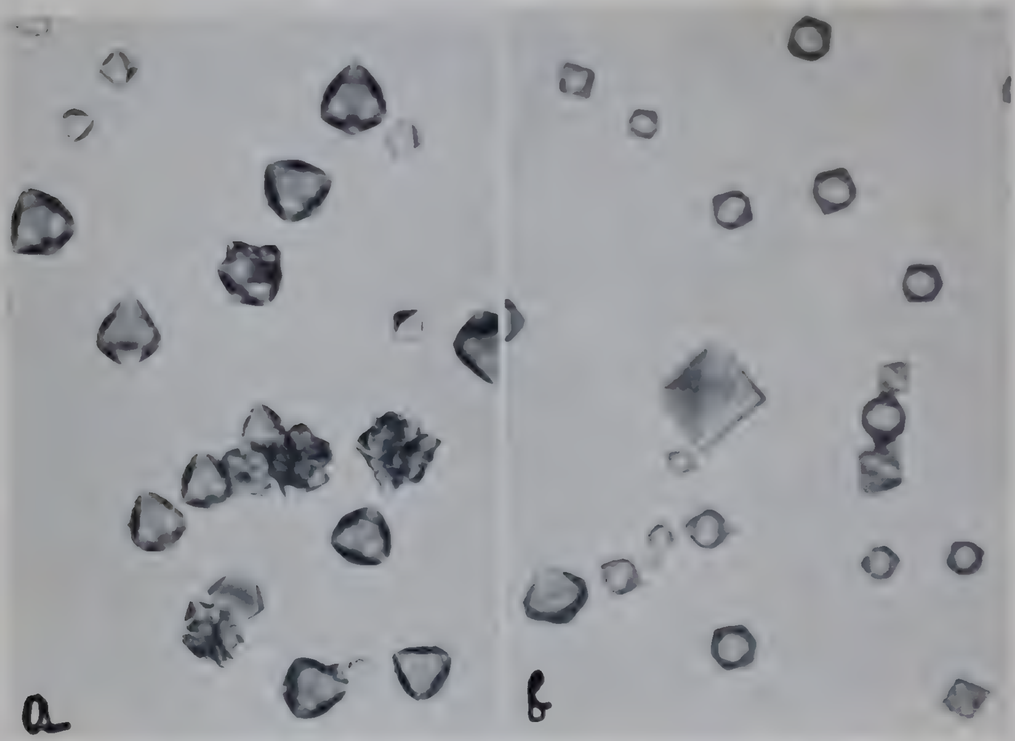


FIG. 1. Crystals (*a*) of dog liver ferritin prepared according to Granick (6) and (*b*) from a highly concentrated dog liver VDM solution.

---

[2] Agner, K., personal communication.

A typical ferritin preparation from horse spleen contained 20.7 per cent iron, 11.0 per cent nitrogen, 1.29 per cent phosphorus, and 0.23 per cent cadmium. Thus, it contained 1.88 mg. of iron per mg. of nitrogen.

The visible light absorption spectra of purified crystalline horse spleen ferritin and a dog liver VDM preparation are shown in Fig. 2. Also shown is the absorption spectrum of a colloidal ferric hydroxide solution prepared by heating a dilute solution of ferric chloride to 100°. The three curves are almost identical when the wave-length is plotted against the extinction coefficient calculated on the basis of iron content.

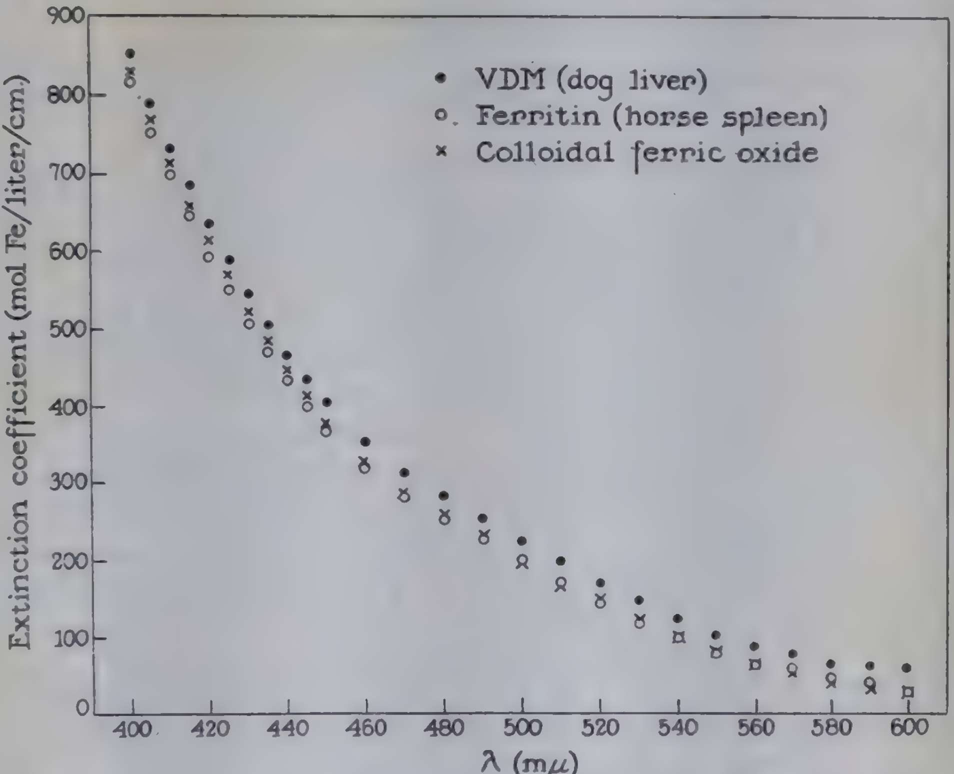


FIG. 2. Visible light absorption spectra of solutions of dog liver VDM, crystalline horse spleen ferritin, and colloidal ferric hydroxide, determined by means of the Beckman spectrophotometer.

Ultracentrifuge determinations[1] were carried out on a preparation of horse spleen ferritin and on a highly concentrated VDM solution prepared from the liver of dogs in the irreversible phase of hemorrhagic shock. The ferritin was purified without the use of $CdSO_4$, by means of alcohol precipitation at low temperatures.[2] The ferritin solution, which contained 2.10 mg. of iron per mg. of nitrogen, was centrifuged at 24,000 R.P.M. The deep brown color of the solution was associated with a rapidly sedimenting component, corresponding to the aggregated apoferritin-iron hydroxide complex as reported by Rothen (7). The VDM solution, which contained 1.17 mg. of iron per mg. of nitrogen, was centrifuged in a similar manner. The deep brown material sedimented at a rate similar to that of the ferritin boundary previously obtained. However, there was present, in addition, an equal amount of a colorless component of a much lower molecular weight, the boundary of which did not quite detach itself from the meniscus after centrifugation for 36 minutes at 24,000 R.P.M. The VDM solution therefore contained an iron-protein fraction with sedimentation characteristics similar to that of ferritin. This fraction constituted approximately one-half of the total protein.

The pure ferritin solutions were active at 0.0005 γ of nitrogen per 0.5 ml. in the test rat. This is equal in physiological activity to the most concentrated of the hepatic VDM preparations (Table III).

In order to determine whether the vasodepressor activity of ferritin was associated with the iron or protein moiety, the activity of iron-free crystalline apoferritin was investigated. The apoferritin was prepared according to Granick and Michaelis (8) and further purified by four precipitations at 50 per cent saturation with ammonium sulfate. The purified apoferritin contained 16.2 per cent nitrogen, 0.05 per cent phosphorus, and 0.16 per cent cadmium. It was free of iron. Apoferritin gave a positive VDM test with 0.0005 γ of nitrogen, a potency similar to that of crystalline ferritin. VDM activity was therefore associated with the protein moiety of ferritin. The VDM activities of a number of ferritin and apoferritin preparations are recorded in Table IV. Two fractions, Nos. 1162A and 1162F, Table IV, were precipitated from a ferritin preparation, No. 1162, by fractionation with varying concentrations of $(NH_4)_2SO_4$. Fraction 1162A, precipitated at 23 per cent saturation with $(NH_4)_2SO_4$, contained more iron (2.20 mg. of iron per mg. of nitrogen), whereas Fraction F, precipitated at 34.6 to 40 per cent saturation with $(NH_4)_2SO_4$, contained less iron (0.93 mg. of iron per mg. of nitrogen) than the original ferritin solution, Preparation 1162 (1.88 mg. of iron per mg. of nitrogen). Also shown are two fractions, Nos. 1162X and 1162Y, prepared by the centrifugation of ferritin Preparation 1162 at 13,000 R.P.M. for 30 minutes at 5° in a Sorvall centrifuge. Fraction 1162X was pipetted from the top of the resultant solution and contained 1.28 mg. of iron per mg. of nitrogen, whereas Fraction 1162Y was recovered from the bottom of the tube as a dark brown pellet and redissolved in water. It contained 2.49 mg. of iron per mg. of nitrogen. All four fractions thus prepared, together with the original ferritin solution from which each had been obtained, had identical VDM activities on the basis of their protein or nitrogen content, although their iron content varied. All gave typical ferritin crystals with $CdSO_4$.

Inactivation of VDM Activity of Ferritin after Aerobic Incubation with

Liver Slices—In order to ascertain the possible identity between ferritin and naturally occurring VDM, a variety of experiments were carried out by means of which such an identity could be established. A characteristic of naturally occurring VDM is its inactivation by normal liver slices on aerobic incubation *in vitro* at 37.5° (9) (Reaction 6, Table I). This is true for VDM in the blood and in saline washes of the liver of dogs in irreversible hemorrhagic shock, and for VDM resulting from the anaerobic incubation of normal liver slices. However, VDM cannot be inactivated by normal liver slices which have been exposed to a previous anaerobic incubation for 2 hours (Reaction 8, Table I).

TABLE IV

VDM Activity of Ferritin and Apoferritin

Source of preparation	Fe content	VDM activity in terms of	
		Nitrogen	Iron
		Ferritin	
	mg. Fe per mg. N	$\gamma \times 10^{-4}$	$\gamma \times 10^{-4}$
Horse spleen, Preparation 1166	1.27	5	6
" " " 4	1.31	5	7
" " " 2	1.73	5	9
" " " 1162	1.88	5	9
" " Fraction 1162F	0.93	5	5
" " " 1162A	2.20	5	11
" " " 1162X	1.28	5	6
" " " 1162Y	2.49	5	12
Dog liver, Preparation 2	1.24	5	6
" " " 3	1.30	5	7
" " " 1	1.68	5	8
Human liver, Preparation 1	1.09	5	5
Horse " " 1	1.22	5	6
		Apoferritin	
Horse spleen, Preparation 1	0	5	0
Dog liver, Preparation 1	0.06	1	0.06
" " " 2	0.04	5	0.2
Human liver, Preparation 1	0.02	5	0.1

A series of experiments was performed, in collaboration with Dr. Zweifach, to determine whether the VDM activity of ferritin could be inactivated under these conditions. The results are presented in Table V. The VDM activity of 0.0005 γ of ferritin nitrogen, which is equivalent to the VDM activity of 0.5 ml. of plasma from dogs in irreversible shock, was completely inactivated by incubation with normal dog liver slices in oxygen.

Inactivation also occurred with twice this concentration, or 0.001 γ of ferritin nitrogen. When the liver slices were kept anaerobic for 2 hours prior to aerobic incubation with ferritin, no inactivation of the VDM activity due to ferritin occurred. In these respects the behavior of ferritin and that of naturally occurring VDM were identical.

Identification of Hepatic VDM with Ferritin by Immunochemical Procedures—In order to provide more specific evidence of the identity of the VDM in concentrated beef and dog liver fractions with ferritin, quantitative immunochemical techniques were employed.[3] Horse spleen ferritin, recrystallized four times and further purified as described previously, was injected intravenously into a group of rabbits in the form of an alum-precipitated suspension containing the equivalent of 0.15 mg. of ferritin nitrogen per ml. The suspension was injected on four consecutive days each week for 4 weeks. The material was administered in graduated doses as follows: 0.5 ml. for 2 days, 1.0 ml. for 2 days, 1.5 ml. for 4 days, 2.0 ml. for 4 days, and 3.0 ml. for 4 days. Serum was obtained 5 days after the last injection. Additional injections were given thereafter in order to maintain and increase the antibody titer of the serum. The quantitative precipitin reaction was performed (10) by the addition of varying amounts of the antigen solution to 1 ml. portions of the antiserum containing enough 0.9 per cent saline to produce a final volume of 3 ml. The mixtures were incubated at 37.5° for 30 minutes and then kept for 48 hours in the refrig-

TABLE V

Inactivation of VDM Activity of Ferritin by Liver Slices

Liver slices were incubated for 2 hours with 5 volumes of Ringer-phosphate at pH 7.4, or with ferritin dissolved in Ringer-phosphate, pH 7.4. Anaerobic liver slices were prepared by incubating for 2 hours with Ringer-phosphate in N_2, washed with cold saline, and then treated with the appropriate solution. The ferritin solution used for incubation contained 0.005 γ of ferritin nitrogen per 0.5 ml. It was tested as such, diluted 1:5 (0.001 γ of ferritin nitrogen per 0.5 ml.) and diluted 1:10 (0.0005 γ of ferritin nitrogen per 0.5 ml.).

Incubation mixture	Vasotropic activity		
	Original solution	Diluted 1:5	Diluted 1:10
Ferritin (control)	VDM	VDM	VDM
Liver slices + ferritin in O_2	"	Neutral	Neutral
Anaerobic liver slices + ferritin in O_2	"	VDM	VDM
" " " + Ringer-phosphate in O_2	" (mild)	Neutral	Neutral

---

[3] We are greatly indebted to Dr. E. A. Kabat, Neurological Institute, New York, for his advice and many details concerning the quantitative precipitin technique.

erator. The total nitrogen of the centrifuged and washed precipitates was determined by the micro-Kjeldahl method. Each supernatant was tested for the presence of excess antibody or antigen by the addition of either antigen or antiserum to aliquots. All analyses were performed in duplicate.

Rabbit antiserum to horse spleen ferritin, which yielded a precipitate with solutions of horse spleen ferritin, also gave precipitates when mixed with concentrated solutions of VDM (Fraction B, Table II) prepared from horse liver. The quantitative data are presented graphically in Fig. 3, which gives the values for the total nitrogen precipitated in the form of an antibody-antigen complex when solutions of ferritin or apoferritin were incubated with the antiserum to ferritin. The curves are identical, indicating that the antibody is directed towards the protein moiety of ferritin and is not influenced by the presence of the bound iron in high concentrations.

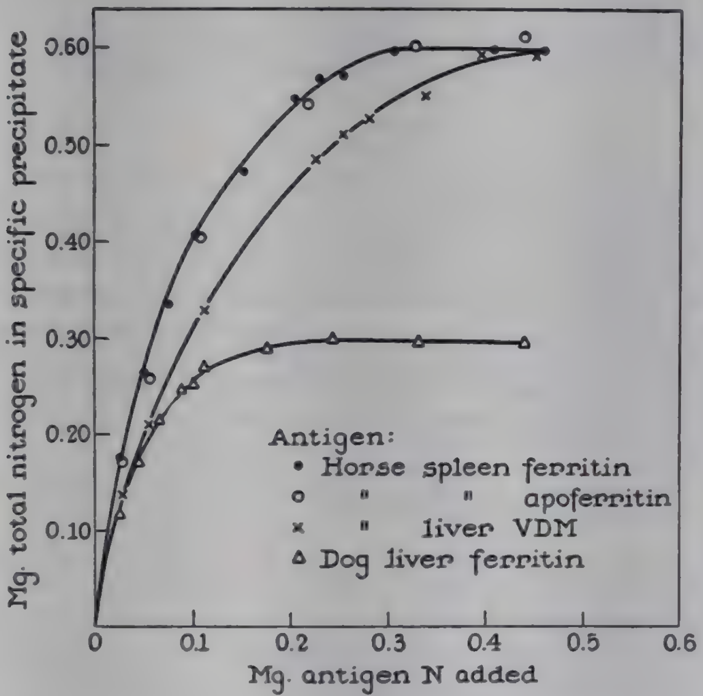


FIG. 3. Quantitative precipitin curves for rabbit antiserum to crystalline horse spleen ferritin.

The curve (Fig. 3) shown for a preparation of horse liver VDM indicates that with equal quantities of ferritin nitrogen and of horse liver VDM nitrogen the total nitrogen in the precipitate obtained with ferritin was greater than that found with the VDM. As a consequence, the early portions of the curves are not superimposable. However, when increasing amounts of horse liver VDM were added, up to the maximal precipitating capacity of the antiserum, maximal precipitation of total nitrogen was obtained at a level corresponding to that obtained with ferritin. Thus, the horse liver VDM solution contained ferritin and in addition some non-ferritin protein which was not precipitable by the antiserum. The quantitative data permit a calculation of the per cent ferritin nitrogen present in the horse liver VDM solution in relation to the total nitrogen content (11). Table VI gives a comparison of the relative amounts of ferritin and VDM nitrogen required to obtain identical amounts of total nitrogen in the precipitates. From this calculation, ferritin nitrogen comprised 65 per cent of the total nitrogen in the horse liver VDM preparation.

Fig. 3 also shows a cross-reaction between ferritin prepared from dog liver and horse spleen ferritin. Maximal precipitation occurred at a much lower level of total nitrogen in the specific precipitates than with horse ferritin. These results indicate that the ferritins of these two species are immunologically related but not identical. However, identical curves have been obtained with horse ferritins from both the liver and spleen, thus establishing their immunological identity.

TABLE VI

Percentage Ferritin in Horse Liver VDM from Immunochemical Data (See Fig. 3)

The VDM activity of the horse spleen ferritin was obtained with 0.0005 γ of nitrogen per 0.5 ml., that for the horse liver VDM solution with 0.001 γ of nitrogen per 0.5 ml. The values for the total N in the specific precipitates were chosen so as to correspond to the portion of the curves where antibody is present in excess.

Total nitrogen in ppt.	Antigen N of ferritin	Antigen N of VDM	Per cent ferritin in VDM
mg.	*mg.*	*mg.*	
0.240	0.044	0.068	65
0.320	0.068	0.108	62
0.360	0.084	0.132	64
0.420	0.112	0.172	65
0.460	0.140	0.204	69
Average			65

Comparable experiments were carried out with dog liver ferritin, a dog liver VDM preparation, and the antiserum obtained from rabbits immunized to crystalline dog liver ferritin. As with the horse spleen ferritin and horse liver VDM, the curve obtained with dog liver VDM was initially lower than that for the crystalline dog liver ferritin, but ultimately rose to a similar maximum. The antiserum to dog liver ferritin gave a cross-reaction with horse spleen ferritin. However, in this instance the curves were reversed, the lower maximum of total nitrogen in the precipitates being given by the horse ferritin. Rabbit antiserum to human liver ferritin gave a positive cross-reaction with horse spleen ferritin.

Identification of Ferritin with Naturally Occurring VDM by Immunological Procedures—The identity of hepatic VDM with ferritin did not preclude the possibility that the VDM activity present in the blood of dogs in irreversible shock might differ from ferritin. This might be approached by the direct isolation of ferritin from the plasma of dogs in shock. However, since the VDM activity in 0.5 ml. of such plasma would be expected to be equivalent to that given by 0.0005 γ of ferritin nitrogen, such an isolation was deemed impractical. Furthermore, addition of antiferritin serum to 0.0005 γ of ferritin nitrogen would not yield a visible precipitate for purposes of identification. For these reasons, the method of bioassay by the rat test was employed in conjunction with immunological procedures.

Preliminary to these experiments, ferritin was precipitated from a concentrated horse liver VDM solution by incubation for 30 minutes at 37.5° with an excess of antiserum to horse spleen ferritin. The precipitate was removed by centrifugation. The filtrate was assayed for VDM activity and found to be neutral. As a control, antiserum alone was tested and also found to be neutral. A similar incubation was then carried out with very low concentrations of horse liver VDM, comparable to that present in plasma from a dog in irreversible shock. No precipitate was obtained but the VDM activity was abolished. Hence, the antibody-antigen complex apparently did not dissociate sufficiently, following its injection, to elicit any vascular response in the rat test. Under similar conditions incubation of VDM with normal rabbit serum left the VDM activity unaffected.

VDM of Plasma from Dogs in Shock—Rabbit antiserum to crystalline dog liver ferritin was incubated with plasma from dogs in the hyporeactive phase of hemorrhagic shock. Under these experimental conditions, which are given in detail in Table VII, the VDM activity of the plasma was abolished. Normal rabbit serum was without effect. Antiserum alone exerted no vasotropic activity in the rat test. Inactivation of VDM in plasma from dogs in shock was likewise achieved by incubation with antiserum to horse spleen ferritin. This is further evidence of the immunological cross-reaction between horse and dog ferritins.

VDM from Anaerobic Liver Slices—VDM was prepared by a 2 hour anaerobic incubation of normal dog liver slices at 37.5° in 5 volumes of Ringer-phosphate solution. The VDM solution was then incubated with antiferritin serum as described above, with a resultant loss of VDM activity.

VDM of Plasma in Chronic Experimental Renal (Dog) and Essential (Human) Hypertension—In previous studies from this laboratory (12) it was observed that during the acute stage of experimental renal hypertension, induced in dogs by the application of the Goldblatt clamp, VEM appeared in the blood. However, after the establishment of a chronic hypertensive state, the blood gave a neutral test by the rat assay method.

This neutral test was found to result from the presence of high concentrations of both VDM and VEM in a ratio which led to mutual neutralization (13). This was demonstrated by the aerobic incubation of such "neutral" plasma with normal kidney slices, a procedure which inactivates VEM but not VDM. Following this inactivation of VEM, the dog plasma gave a strongly positive VDM reaction.

To complement this study, plasma samples from dogs with chronic renal hypertension, which gave "neutral" tests, were now incubated with antiserum to crystalline dog ferritin. Following incubation, these plasmas exerted pronounced VEM effects, indicating the unmasking of VEM through the formation of an inert VDM-antibody complex (Table VII).

TABLE VII

Inactivation of Naturally Occurring VDM by Antiferritin Serum

The VDM solution was tested for vasotropic activity after incubation of 2 ml. of the solution with 1.0 ml. of saline at 37.5° for 30 minutes. Another 2 ml. aliquot of the VDM solution was incubated in the same manner with 1.0 ml. of an antiserum to the appropriate ferritin (dog or human).

The hypertensive dog plasma was obtained from dogs made hypertensive by removal of one kidney and partial clamping of the renal artery of the remaining kidney. Hypertensive human plasma was obtained from patients with chronic essential hypertension.

Source of VDM	Vasotropic activity	
	Incubation with saline	Incubation with antiserum
Dog plasma, irreversible shock	VDM	Neutral
" liver slices incubated in N_2	"	"
Hypertensive dog plasma	Neutral	VEM
" human plasma	"	"

In cases of chronic essential hypertension in man, plasma was likewise found to give a "neutral" reaction which was converted to a strong VDM effect after aerobic incubation with normal kidney slices. The incubation of rabbit antiserum to crystalline human ferritin with plasma from such patients led to the appearance of a strong VEM reaction, indicating the removal of VDM. By way of control, similar studies were carried out with plasma from normotensive dogs and humans which are characterized by their neutral effect in the rat test. Neutral tests were also obtained after incubation of such plasmas with antiferritin serum.

DISCUSSION

Experiments have been described which led to the identification as ferritin of a hepatic vasodepressor, previously referred to as VDM. This was accomplished by a combination of chemical and immunochemical procedures, together with the utilization of the rat mesoappendix test of Zweifach *et al.* (2).

In the course of this study, the question arose as to which portion of the ferritin molecule was responsible for its vasotropic effects. Apoferritin is a very homogeneous protein. Ferritin, on the other hand, is not a definite molecular species, but, as shown by the ultracentrifuge studies of Rothen (7), consists of a mixture of apoferritin-iron hydroxide (ferritin) and apoferritin. In the ferritin preparation studied by Rothen, apoferritin was present to the extent of 25 per cent. Results in our laboratory are confirmatory of Rothen's findings. Thus, we were able to separate chemically a crystalline ferritin preparation (No. 1162, Table IV) into a number of fractions in which the iron content varied from 0.93 to 2.20 mg. of iron per mg. of nitrogen. A similar fractionation of Preparation 1162 was accomplished by high speed centrifugation (Fractions 1162X and 1162Y, Table IV). When the various ferritin preparations with different iron to nitrogen ratios were compared with respect to VDM activity, the correlation of VDM activity with nitrogen content was excellent, whereas there was a poor correlation between activity and iron content. Indeed, on the basis of nitrogen content, apoferritin, which was devoid of iron, proved as active as ferritin. It is therefore the protein moiety of ferritin which is responsible for its vasodepressor activity.

The next problem which arose concerned the identity of the naturally occurring vasodepressor materials in the blood during the irreversible stage of hemorrhagic shock and in the chronic stage of experimental renal and human essential hypertension. The fractionation procedures which were evolved for the preparation of active VDM solutions were entirely dependent on the vascular changes observed in the rat test. Although this test could reveal a specific type of vasodepressor activity, there was no certainty that this vascular response was characteristic of only one substance in the body. Thus, the isolation of a single compound from the liver (ferritin) with VDM activity did not necessarily mean that the vasodepressor in the blood of dogs in irreversible shock was identical with or even related to it. Similar problems have arisen in the past with no direct solution; *e.g.*, the relation of the epinephrine-like substances in blood to epinephrine isolated from the adrenals.

Because of the protein nature of the ferritin-apoferritin complex, this uncertainty could be resolved by immunochemical methods. The specific combination of ferritin-antibody with ferritin in dilute solutions, together with the rat test for VDM activity, made it possible to determine whether any naturally occurring VDM was identical with ferritin. This was found to be the case for VDM in liver of dogs in irreversible shock, and for VDM in blood both during the irreversible stage of experimental hemorrhagic shock and in the chronic stage of experimental renal and essential human hypertension. However, this procedure does not make it possible to determine whether it is ferritin or apoferritin which is responsible for VDM activity in these cases, because of the absence of a visible precipitate at such low concentrations of antigen. When larger amounts of these substances are present, as in hepatic concentrates, such a differentiation is possible, on the basis of the iron content of the specific precipitate formed when the antibody precipitates the ferritin from solution.

Of particular interest are the immunological cross-reactions between horse and human and between horse and dog ferritin. These cross-reactions have interesting physiological implications. Large quantities of antibody to horse, dog, rat, and human ferritins have been prepared and are being utilized for experiments on animals and man with a view to the elucidation and possible modification of the variety of conditions associated with derangements of the VDM-VEM mechanisms. These studies, which are being carried out with Dr. Zweifach and Dr. Baez of this laboratory, will be the subject of a separate report.

It is beyond the scope of this paper to discuss in detail the physiological rôle of ferritin in the regulation of the peripheral circulation. The participation of VDM and VEM in experimental shock, hypertension, and nutritional cirrhosis has been dealt with in other papers (1, 14, 15). On the basis of these studies, it has been postulated that VDM and VEM are oppositely acting components of a homeostatic mechanism for the regulation of the peripheral circulation. In addition, it has been found that VDM exerts a profound antidiuretic effect (16) in the dog and rabbit by inducing an increased tubular resorption of water. This is a phenomenon of particular interest in relation to the antidiuresis which is observed in hepatic cirrhosis.

Hitherto, the only function of the ferritin-apoferritin system appeared to be that of iron transport and storage (17). To this may now be added the newly described and important function of participation in the regulation of the peripheral circulation. Further study will be required to establish the exact mechanism and significance of this function of ferritin.

The authors would like to express their indebtedness to Delilah Metz, Ruth Jacob, and Vera Bergman for carrying out the rat assays in these studies and to Dr. B. W. Zweifach and Dr. R. F. Furchgott of this laboratory for their many valuable suggestions in the course of this work.

SUMMARY

A hepatic vasodepressor principle (VDM) which has been found to participate in the regulation of the peripheral circulation has been con-

centrated from saline extracts of anaerobic beef, dog, horse, and human liver. The VDM activity of these concentrates, as measured by the effects induced on the terminal vascular bed, was proportional to their ferritin content. Crystalline ferritin and its iron-free component, apoferritin, were found to exert similar vascular effects.

By immunochemical procedures the VDM of hepatic concentrates was identified as ferritin. By similar procedures, naturally occurring VDM, present in the liver and blood of dogs in irreversible shock and in the blood during the chronic stage of experimental (dog) and essential (human) hypertension, was identified with ferritin or apoferritin.

These findings indicate that, in addition to iron transport and storage, the ferritin-apoferritin system plays an important rôle in the regulation of the peripheral circulation.

BIBLIOGRAPHY

1. Shorr, E., Zweifach, B. W., and Furchgott, R. F., *Science,* **102,** 489 (1945).
2. Chambers, R., Zweifach, B. W., Lowenstein, B. E., and Lee, R. E., *Proc. Soc. Exp. Biol. and Med.,* **56,** 127 (1944).
3. Laufberger, V., *Bull. Soc. chim. biol.,* **19,** 1575 (1937).
4. Mazur, A., and Shorr, E., *Federation Proc.,* **6,** 275 (1947).
5. Granick, S., *Chem. Rev.,* **38,** 379 (1946).
6. Granick, S., *J. Biol. Chem.,* **149,** 157 (1943).
7. Rothen, A., *J. Biol. Chem.,* **152,** 679 (1944).
8. Granick, S., and Michaelis, L., *J. Biol. Chem.,* **147,** 91 (1943).
9. Furchgott, R. F., and Shorr, E., *Federation Proc.,* **6,** 108 (1947).
10. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.,* **50,** 809 (1929).
11. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.,* **55,** 555 (1932). Heidelberger, M., and Kabat, E. A., *J. Exp. Med.,* **63,** 737 (1936). Kabat, E. A., and Glusman, M., *Am. J. Med.,* **4,** 653 (1948). Kabat, E. A., and Mayer, M. M., Experimental immunochemistry, Springfield (1948).
12. Zweifach, B. W., Baez, S., and Shorr, E., *Federation Proc.,* **6,** 232 (1947).
13. Shorr, E., Zweifach, B. W., Furchgott, R. F., and Baez, S., *Federation Proc.,* **6,** 200 (1947).
14. Shorr, E., Zweifach, B. W., Furchgott, R. F., and Baez, S., *Tr. Assn. Am. Physn.,* **60,** 28 (1947).
15. Baez, S., Zweifach, B. W., Mazur, A., and Shorr, E., *Proc. Soc. Exp. Biol. and Med.,* **64,** 154 (1947).
16. Baez, S., Mazur, A., and Shorr, E., *Federation Proc.,* **7,** 5 (1948).
17. Hahn, P. F., Granick, S., Bale, W. F., and Michaelis, L., *J. Biol. Chem.,* **150,** 407 (1943). Granick, S., and Hahn, P. F., *J. Biol. Chem.,* **155,** 661 (1944).

DIMETHYLTHETIN AND DIMETHYL-β-PROPIOTHETIN IN METHIONINE SYNTHESIS*

By JACOB W. DUBNOFF and HENRY BORSOOK

(*From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena*)

(Received for publication, May 5, 1948)

In a previous communication it was shown that choline and betaine are effective in promoting methionine synthesis from homocysteine in tissue homogenates (1). Data presented in this paper indicate that dimethylthetin, $(CH_3)_2{}^+ SCH_2COO^-$, which has been shown by Welch (2) to be lipotropic and has been reported by du Vigneaud (3) to promote growth on a methionine-free, homocysteine-containing diet, is 20 times as active as betaine in methionine formation. Dimethyl-β-propiothetin, $(CH_3)_2{}^+S(CH_2)_2COO^-$, recently isolated from *Polysiphonia fastigiata* by Challenger and Simpson (4) is also highly active. The enzyme for this transmethylation is found in the liver and kidney of all animals tested. Its high activity and general distribution suggest its biological importance in methionine synthesis.

Methods

Viobin extracts were prepared by stirring 5 gm. of Viobin[1] in 100 ml. of water for half an hour and filtering. Fresh extracts were prepared from organs of animals which had been killed by stunning and thoroughly bled. The organs were chilled, homogenized with 2 parts of buffer in the homogenizer of Potter and Elvehjem (5), and strained through cheese-cloth. The buffer (6) is composed of 0.0128 M sodium phosphate, pH 7.4, 0.123 M sodium chloride, 0.005 M potassium chloride, and 0.003 M magnesium sulfate.

Methylmercaptoacetic acid was prepared by Dr. M. Fling according to the method of Larsson (7).

Dimethylthetin was kindly given us by Dr. A. D. Welch.

Dimethyl-β-propiothetin was prepared by the method of Biilmann and Jensen (8).

DL-Homocystine was prepared from DL-methionine by the method of

---

* Presented before the meeting of the American Society of Biological Chemists at Atlantic City, March 15-19, 1947. Aided by a grant from the United States Public Health Service.

[1] Viobin preparations are commercially prepared desiccated and defatted raw tissues manufactured by the Viobin Corporation, Monticello, Illinois.

Butz and du Vigneaud (9) and reduced to DL-homocysteine as described by Riegel and du Vigneaud (10).

Dimethylglycine was prepared by the method of Schubert (11).

4 ml. of buffer solution containing the enzyme and substrates were incubated in 20 ml. beakers in an apparatus especially designed for nonmanometric studies (12). In this apparatus as many as thirty 20 ml. beakers employed as reaction vessels are held in a stainless steel container fitted with a cover through which any gas mixture may be passed. The container is incubated at 38° and shaken at 90 oscillations per minute in a small water bath.

After incubation the solutions were deproteinized by the addition of 0.5 ml. of 30 per cent trichloroacetic acid and 1 ml. of water to each beaker. With guinea pig liver homogenates after the addition of trichloroacetic acid, the solutions were brought to a boil in the incubation apparatus in order to get filtrates which would remain clear in the analytical procedure. Filtrates of other organs offered no difficulty.

Methionine was determined by a modification of the method of McCarthy and Sullivan (13). The procedure was as follows: To 2 ml. of the trichloroacetic acid filtrate were added 0.2 ml. of 5 N NaOH followed by 0.1 ml. of 1 per cent freshly made sodium nitroprusside. The solutions were incubated at 38° for 5 to 10 minutes and then 1 ml. of an acid mixture consisting of 9 volumes of concentrated hydrochloric acid and 1 volume of 85 per cent phosphoric acid was added. After 10 minutes the solutions were read in a Klett-Summerson colorimeter with a green filter.

If the solutions are cooled in ice before the addition of strong acid, homocysteine reduces the color by an amount which increases with increasing concentration of methionine. When this cooling step is omitted as described, the decrease in color due to homocysteine is a small and constant value over a wide range of methionine concentrations, and no difficulty is encountered with gas bubbles during the measurement of the color. 1 mole of methylmercaptoacetic acid formed by the demethylation of dimethylthetin gives a color equivalent to 0.6 mole of methionine in this determination. Accordingly, all apparent increases of methionine due to the addition of dimethylthetin must be divided by 1.6 to compensate for the equivalent amount of methylmercaptoacetic acid formed in the reaction. Dimethyl-β-propiothetin is chromogenic, but it can be destroyed by allowing the solutions to stand overnight after the addition of 5 N NaOH. The nitroprusside is added the following day and the determination continued as described. The chromogenic power of methylmercaptopropionic acid, the demethylated product of dimethyl-β-propiothetin, is approximately 0.9 that of methionine on a mole basis.

Results

In rat liver choline, betaine, dimethyl-β-propiothetin, and dimethylthetin show significant activity in methionine formation (Table I). Dimethylethanolamine, dimethylglycine, and methylmercaptoacetic acid, the compounds formed by the removal of one methyl group from choline, betaine, and the dimethylthetin, respectively, are inactive.

If homocysteine is present in excess, it can be directly shown that only one methyl group is transferred per mole of dimethylthetin (Fig. 1). The reaction with betaine is too slow to reach equilibrium; only about 0.6 mole equivalent of methyl is transferred per mole of betaine in 24 hours.

TABLE I

Methionine Formation in Rat Liver Homogenate

1 ml. of 1:4 homogenate. Homocysteine 25 mg. per cent; all other substrates 12.5 mg. per cent. Total volume 4 ml.; gas phase, nitrogen; time, 3 hours; temperature, 38°. Methionine values are the average of three determinations.

Reaction mixture	Methionine found
	mg. per cent
Homocysteine	1.0 ± 0.05
" + choline	2.6 ± 0.05
" + betaine	4.2 ± 0.01
" + dimethyl-β-propiothetin	4.5* ± 0.1
" + dimethylthetin	8.4* ± 0.05
" + methylmercaptoacetic acid	1.0 ± 0.0
" + dimethylglycine	1.0 ± 0.1
" + dimethylethanolamine	1.1 ± 0.05
" + monomethylethanolamine	1.3 ± 0.2

* Corrected for chromogenic value of demethylated product as indicated in section on methods.

Table II shows the distribution of the betaine- and dimethylthetin-transmethylating enzymes and approximate $Q_{\text{methionine}}$ values for various tissues. Only liver and kidney show activity with betaine and dimethylthetin.

The effect of pH on the activity of the dimethylthetin enzyme is shown in Fig. 2. The optimum pH is about 7.8.

Neither the betaine nor the dimethylthetin enzyme is inactivated by simple dialysis.

The formation of methionine from dimethylthetin, dimethyl-β-propiothetin, betaine, or choline was not inhibited by cyanide, azide, arsenate, or arsenite.

The two enzymes can be distinguished by the greater stability of the dimethylthetin enzyme at pH 4.0 as shown in Fig. 3. The variation of the ratio of $Q_{\text{dimethylthetin}}$ to Q_{betaine} (Table II) also points to two different enzymes for the two methylators.

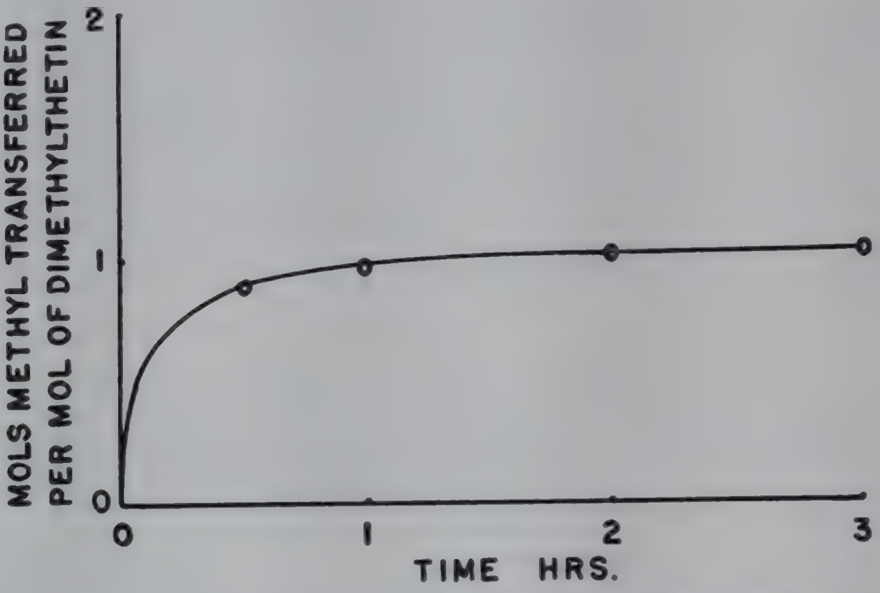


FIG. 1. Number of methyl groups transferred per mole of dimethylthetin. 1 ml. of 5 per cent solution of Viobin liver in buffer (6); dimethylthetin 5 mg. per cent; L-homocysteine 12.5 mg. per cent. Temperature, 38°; gas phase, nitrogen.

TABLE II

Distribution and Activity of Methionine-Forming Enzyme Systems

The figures are $Q_{\text{methionine}}$; average of two determinations; probable error ±5 per cent.

Each vessel contained 1 ml. of homogenized guinea pig or rat tissue diluted 1:4, or 1 ml. of a 5 per cent Viobin solution. DL-Homocysteine 25 mg. per cent, betaine or dimethylthetin 12.5 mg. per cent. Total volume, 4 ml.; gas phase, nitrogen; time, 3 hours; temperature, 38°.

	Guinea pig			Rat			Hog (Viobin)		
	Dimethylthetin	Betaine	Dimethylthetin / Betaine	Dimethylthetin	Betaine	Dimethylthetin / Betaine	Dimethylthetin	Betaine	Dimethylthetin / Betaine
Liver	1.5	0.09	17	1.5	0.11	14	1.3	0.14	9
Kidney	0.3	0.06	5	0.2	0.03	7	0.9	0.03	30
Spleen							0	0	
Muscle	0	0							
Pancreas							0	0	

The dimethylthetin enzyme may be purified free of betaine enzyme and concentrated about 100-fold by precipitating a 5 per cent aqueous solution of Viobin[1] liver with alcohol. The fraction precipitated at between 11.5 and 18 per cent alcohol contains almost all the activity of the whole extract. While the crude homogenate is effective with homocystine and

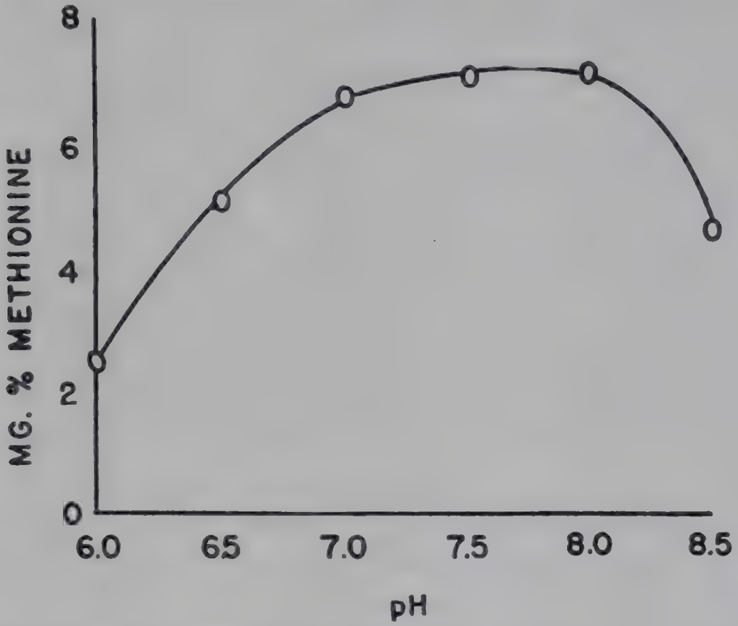


FIG. 2. Effect of pH on methionine formation from dimethylthetin. 1 ml. of 5 per cent Viobin; dimethylthetin 12.5 mg. per cent, DL-homocysteine 12.5 mg. per cent. Temperature 38°; gas phase, nitrogen; time, 1 hour.

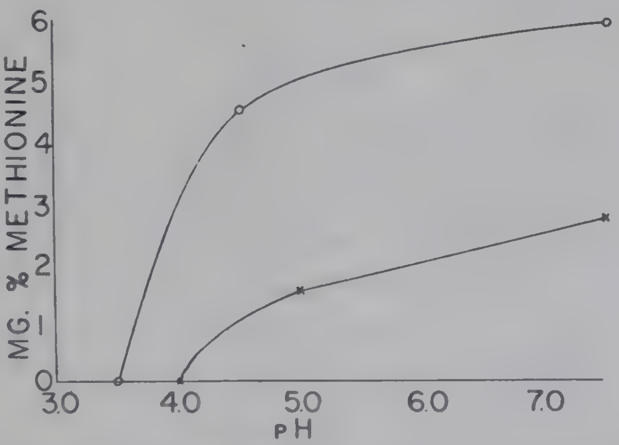


FIG. 3. Stability of enzyme to pH. Upper curve, dimethylthetin transmethylase; lower curve, betaine transmethylase. 5 per cent Viobin allowed to stand 24 hours at 5° at given pH, and relative activity at pH 7.5 determined at 38°. Substrates 12.5 mg. per cent. Gas phase, nitrogen; total volume, 4 ml.; time, 3 hours.

homocysteine, this fraction reacts only with homocysteine. Although the dimethylthetin transmethylase can be prepared free from the betaine transmethylase by further alcohol fractionation, all active betaine transmethylase preparations have had dimethylthetin transmethylase activity.

Whether the latter enzyme is required for betaine activity is still uncertain.

An attempt was made to demonstrate the presence of dimethylthetin in animal tissues. Neutral and acidified aqueous and alcoholic extracts of Viobin[1] pancreas, spleen, kidney, and liver, fresh beef pancreas, and guinea pig and rat liver and kidney were inactive with a partially purified dimethylthetin enzyme. Liver and kidney homogenates of the rat and guinea pig were allowed to stand at pH 4.0 at 5° for 18 hours to destroy all but the dimethylthetin methylating system. Any increase in methionine on addition of homocysteine in such a system could be considered as evidence of preformed dimethylthetin. No increase in methionine occurred. These experiments seem to exclude any significant quantity of preformed dimethylthetin in these tissues. On the addition of labile methyl donors to homogenates there is a slight synthesis of methionine in most experiments, suggesting the presence of small amounts of preformed homocysteine.

None of these reactions is reversible under our conditions; *i.e.*, methionine will not remethylate dimethylglycine, dimethylethanolamine, or mercaptoacetic acid aerobically or anaerobically in the presence of high energy-yielding metabolites. These reactions were studied by measuring the change in methionine concentration in the presence of these putative methyl acceptors.

Although dimethylthetin is very effective with homocysteine, it will not methylate glycocyamine in rat or guinea pig liver homogenates.

DISCUSSION

Evidence has been presented that there are at least four compounds which can furnish methyl groups for methionine synthesis in tissue homogenates. These compounds, dimethylthetin, dimethyl-β-propiothetin, betaine, choline, are all "onium" compounds characterized by the coordination of an additional methyl group to sulfur or nitrogen, and they all react in the absence of oxygen or energy donors. It has been directly demonstrated in these and previous studies (1) that the methyl groups in dimethylglycine, dimethylethanolamine, and methylmercaptoacetic acid are not transferred under conditions in which a methyl of the "onium" compound is labile. This confirms the findings in feeding experiments on the availability of methyl in dimethylglycine and dimethylaminoethanol (14).

The methyl of methionine, which is held by a covalent bond to sulfur, but is nevertheless labile, requires energy for its transfer to glycocyamine (15) and to nicotinamide (16).

Du Vigneaud and his collaborators have proved rigorously that the methyl groups of choline, betaine, and methionine constitute a dietary "pool" of physiologically interchangeable methyl groups. The evidence reported here indicates that in the tissues there does not exist a "pool" of labile methyl groups in the sense that the transfer is directly between any two members of the dietary pool of labile methyl compounds. It seems likely that rather than a "pool" there are specific methyl donors for each methyl acceptor (*i.e.*, the methylation of glycocyamine by methionine (15)), and that a given methyl compound may be related to another only indirectly through a series of methyl transfer reactions.[2]

We have so far failed to find any methyl transfer reaction which is reversible in the usual chemical sense. Methionine, for example, does not directly remethylate dimethylethanolamine, dimethylglycine, or methylmercaptoacetic acid.

The present findings indicate that the physiological transfer from methionine to choline must be a cyclic process in which some and possibly all steps are irreversible. In some stages of the cycle oxidation and presumably, therefore, energy is required; other stages may proceed anaerobically. In such a dynamic state a given labile methyl-containing compound cannot be utilized or depleted to the same extent by all methyl acceptors. This is in accord with the fact that not all methyl donors are equally effective in overcoming growth inhibition by such compounds as glycocyamine (17, 18) and nicotinamide (19, 20).

The effectiveness of dimethylthetin suggests its importance in the biological synthesis of methionine. Its apparent absence from animal tissues may be due to its rapid demethylation in the presence of homocysteine, or it may be present and function in only catalytic amounts. The occurrence of dimethylthetin in the dietary sources has not been studied. Its homologue, dimethyl-β-propiothetin, has been isolated from algae (4) and may be present in pineapple (21). This compound may, therefore, prove to be more important biologically than dimethylthetin despite its lower activity in methionine synthesis.

The authors wish to acknowledge the assistance in this work of Miss I. Silberbach.

SUMMARY

1. An enzyme has been isolated in a partially purified state which transfers a methyl group from either dimethylthetin or dimethyl-β-propiothetin to homocysteine.

---

[2] The data presented here do not establish a direct methyl transfer from all four compounds to homocysteine. Unpublished evidence suggests that choline is first oxidized to betaine. Whether the latter compound transfers directly or through a methylthetin derivative has not yet been determined.

2. Dimethylthetin is 10 to 20 times as effective as betaine as a methyl donor in tissue homogenates. Dimethyl-β-propiothetin is more effective than betaine.

3. The enzyme is found in the liver and kidney of rat, guinea pig, and hog, but is absent from muscle, pancreas, and spleen.

4. The reaction proceeds until one methyl group has been transferred from dimethylthetin to homocysteine. Mercaptoacetic acid is inactive.

5. Homocystine is completely inactive as a methyl acceptor in purified extracts.

6. The reaction is independent of O_2 and is not inhibited by oxidative poisons.

7. The dimethylthetin transmethylase is distinguished from the betaine transmethylase by its stability at pH 4.5.

8. The possible rôle of dimethylthetin and dimethyl-β-propiothetin in the biological formation of methionine is discussed.

BIBLIOGRAPHY

1. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **169**, 247 (1947).
2. Welch, A. D., private communication to Moyer, A. W., and du Vigneaud, V., *J. Biol. Chem.*, **143**, 373 (1942).
3. du Vigneaud, V., *Harvey Lectures*, **38**, 39 (1942–43).
4. Challenger, F., and Simpson, M., *Biochem. J.*, **41**, p. xl (1947).
5. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
6. Cohen, P. P., and Hayano, M., *J. Biol. Chem.*, **166**, 239 (1946).
7. Larsson, E., *Ber. chem. Ges.*, **63**, 1347 (1930).
8. Biilmann, E. I., and Jensen, K. A., *Bull. Soc. chim.*, **3**, 2306 (1936).
9. Butz, L. W., and du Vigneaud, V., *J. Biol. Chem.*, **99**, 135 (1932–33).
10. Riegel, B., and du Vigneaud, V., *J. Biol. Chem.*, **112**, 149 (1935–36).
11. Schubert, M. P., *J. Biol. Chem.*, **111**, 671 (1935).
12. Dubnoff, J. W., *Arch. Biochem.*, **17**, 327 (1948).
13. McCarthy, T. E., and Sullivan, M. X., *J. Biol. Chem.*, **141**, 871 (1941).
14. du Vigneaud, V., Chandler, J. P., Simmonds, S., Moyer, A. W., and Cohn, M., *J. Biol. Chem.*, **164**, 603 (1946).
15. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **132**, 559 (1940); **171**, 363 (1947).
16. Perlzweig, W. A., Bernheim, M. L. C., and Bernheim, F., *J. Biol. Chem.*, **150**, 401 (1943).
17. du Vigneaud, V., Chandler, J. P., Cohn, M., and Brown, G. B., *J. Biol. Chem.*, **134**, 787 (1940).
18. Stetten, D., Jr., and Grail, G. F., *J. Biol. Chem.*, **1** , 175 (1942).
19. Handler, P., and Dann, W. J., *J. Biol. Chem.*, **146**, 357 (1942).
20. Stekol, J., Abstracts, Biological Division, American Chemical Society, Memphis, B9 (1942).
21. Haagen-Smit, A. J., Kirchner, J. G., Deasy, C. L., and Prater, A. N., *J. Am. Chem. Soc.*, **67**, 1651 (1945).

BIOPHYSICAL STUDIES OF BLOOD PLASMA PROTEINS

X. FRACTIONATION STUDIES OF NORMAL AND IMMUNE HORSE SERUM*

By H. F. DEUTSCH and J. C. NICHOL†

(From the Department of Physiological Chemistry and the Department of Chemistry, University of Wisconsin, Madison)

(Received for publication, June 19, 1948)

A large portion of the antibody proteins of horse serum resides in a fraction possessing an electrophoretic mobility between the normal serum γ-globulins and the lipide-rich β-globulins (1–6). While this component has been designated β-globulin by Kekwick and Record (3) and has been shown to consist of two components (β_1 and β_2), it is analogous to the T component of van der Scheer and Wyckoff (4). We have described the corresponding antibody-rich protein fraction from normal human plasma as γ_1-globulin to distinguish it from the normal serum γ_2-globulin (7). We shall retain this terminology. The need for the separation of these two globulins in order that both their biological and physicochemical properties may be elucidated is evident.

Recently Smith and Gerlough (8) applied the low temperature ethanol fractionation procedures of Cohn *et al.* (9) developed for the fractionation of human plasma to the separation of the tetanus antitoxin from the plasma of hyperimmunized horses. They found the antitoxic activity to be associated with various fractions and concluded that the pepsin digestion methods of antibody recovery (10, 11) were more suitable for the concentration of such immune plasma systems than the ethanol type of fractionation. Other work on the ethanol fractionation of various animal sera (12–15) has indicated that the successful separation of any electrophoretically well defined protein entity from a given animal serum will require specific conditions and that the methods designed for human plasma cannot be applied to other animal plasmas *in toto*.

We have found it possible to develop conditions whereby the antibody content of the serum of hyperimmunized horses may be separated in high yield by the low temperature ethanol method in a single precipitation step. This fraction may, however, be divided into various electrophoretic components by subsequent refractionations. The methods of obtaining such

---

* This work was supported in part by grants from the Wisconsin Alumni Research Foundation, Eli Lilly and Company, and the United States Public Health Service.

† Eli Lilly and Company Research Fellow.

fractions from normal and hyperimmunized horses and a description of certain of their biological and physicochemical properties form the basis of this report.

EXPERIMENTAL

Plasma or serum of normal and hyperimmunized horses was used as the source material.[1] The immune serum samples were usually aliquots of relatively large pools of antiserum to either tetanus or diphtheria antitoxin. In addition a pooled serum sample of two horses that had each been immunized simultaneously with diphtheria, tetanus, and a heterologous gas gangrene (vibrion septique and *Bacillus welchii*) toxoid, and *Hemophilus pertussis* and formalized pneumococcus type III vaccines was also studied. While it was realized that type III pneumococcus vaccine is a poor antigen, it was used because the type-specific polysaccharide is nitrogen-free and allows for the ready determination, by quantitative precipitation, of the antibody produced. Plasma samples were defibrinated by the addition of sufficient calcium ion to permit clotting, followed by stirring to remove the fibrin formed. These sera were then fractionated by means of the aqueous ethanol precipitation techniques and as usual temperature, pH, protein concentration, alcohol concentration, and ionic strength were carefully controlled. The fractionation experiments were evaluated in terms of electrophoretic composition and of protein and antibody yields resulting from controlled variations of the several variables of fractionation. All electrophoretic experiments at pH 8.6 were carried out in veronal buffer of ionic strength 0.1 for 9000 seconds at a constant potential gradient of 6.0 to 6.5 volts per cm. The mobility experiments were performed in solutions of ionic strength 0.1 in which sodium chloride made up 80 per cent of the ionic strength, the remainder being the contribution of a univalent buffer salt. Velocity sedimentation analyses were carried out with 0.7 per cent protein solutions in the oil turbine ultracentrifuge at 220,000 times gravity, a schlieren optical method being used to record the position of the moving boundaries as a function of time.[2]

The antibody assays were obtained by the following tests.[3] Preliminary diphtheria antitoxin titers were obtained by Ramon flocculation and final values by guinea pig intracutaneous (L+) tests. The antibodies to *Bacillus tetanus*, vibrion septique, and *Bacillus welchii* toxoids were assayed by the standard mouse tests. The antibody to *Hemophilus pertussis* was determined by agglutination procedures. Pneumococcus antibody assays were attempted by agglutination, by the capsular swelling or *Quellung* reaction, and by precipitin tests with the specific polysaccharide.

---

[1] All of the horse serum samples were supplied through the courtesy of Eli Lilly and Company.

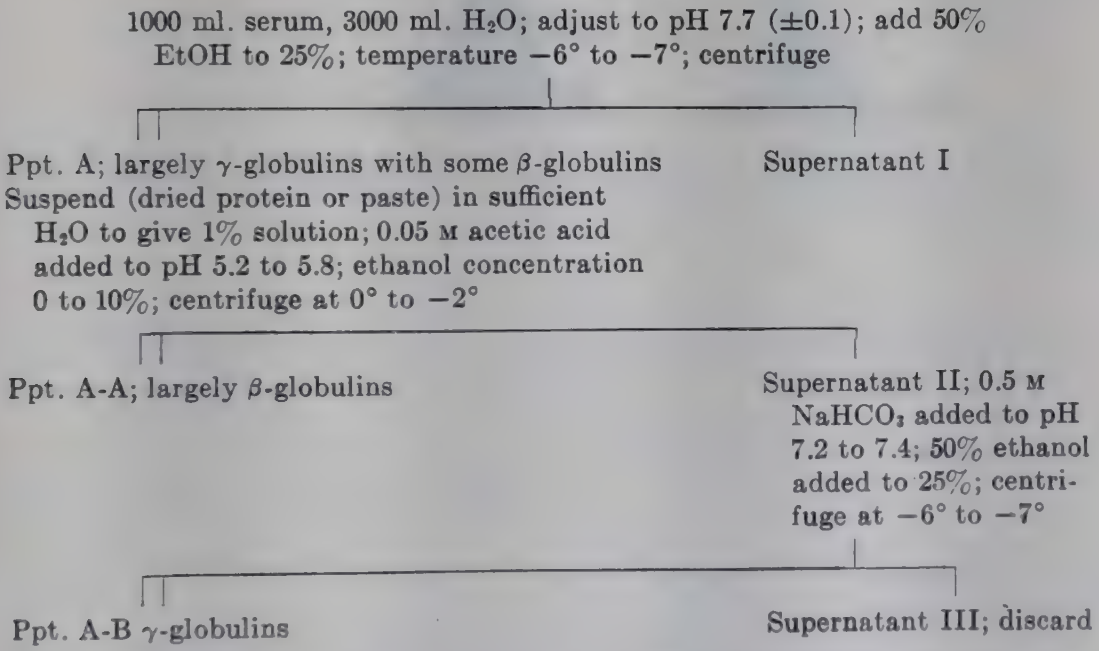
[2] The sedimentation velocity experiments were performed by Mr. E. M. Hanson.

[3] These assays were carried out in the laboratories of Eli Lilly and Company, Indianapolis, Indiana.

Fractionation Results

Preliminary experiments indicated that the electrophoretically heterogeneous γ-globulins and associated antibodies of horse serum could be removed almost quantitatively by precipitation with 25 per cent ethanol at pH 7.5 to 7.8. In addition to the γ-globulins these initial precipitates contained from 5 to 15 per cent of β-globulins. Subsequent experiments were carried out to remove these β-globulins and to provide a γ-globulin fraction made up of proteins having two electrophoretic constituents, the one described as γ_1-globulin and the other as γ_2-globulin. The fractionation conditions which evolved for this purpose are shown in the accompanying Diagram 1.

DIAGRAM 1



1000 ml. serum, 3000 ml. H_2O; adjust to pH 7.7 (±0.1); add 50% EtOH to 25%; temperature −6° to −7°; centrifuge

Ppt. A; largely γ-globulins with some β-globulins
Suspend (dried protein or paste) in sufficient H_2O to give 1% solution; 0.05 M acetic acid added to pH 5.2 to 5.8; ethanol concentration 0 to 10%; centrifuge at 0° to −2°

Supernatant I

Ppt. A-A; largely β-globulins

Supernatant II; 0.5 M $NaHCO_3$ added to pH 7.2 to 7.4; 50% ethanol added to 25%; centrifuge at −6° to −7°

Ppt. A-B γ-globulins

Supernatant III; discard

The electrophoretic diagrams of a typical series of fractions employing antidiphtheritic horse serum as starting material are shown in Fig. 1. From 80 to 100 per cent of the serum antibodies to diphtheria and tetanus toxins are usually found in Precipitate A. Considerable care must be taken to keep all precipitates as cold as possible during their removal and suspension prior to lyophilization to prevent marked destruction of antibody. Occasionally low yields (50 to 60 per cent) are experienced in the initial precipitate but, since the antibody is not found in the supernatant it appears that such marked losses are due to an improper handling of Precipitate

A, resulting in antibody destruction. In Table I are shown the results of various typical fractionations on diphtheria and tetanus antisera in terms of yields of protein and antibody. The antibody recovery data must be considered in relation to the shortcomings of the assay procedures. The data indicate that the major portion of the antibody is recovered in Precipitate A.

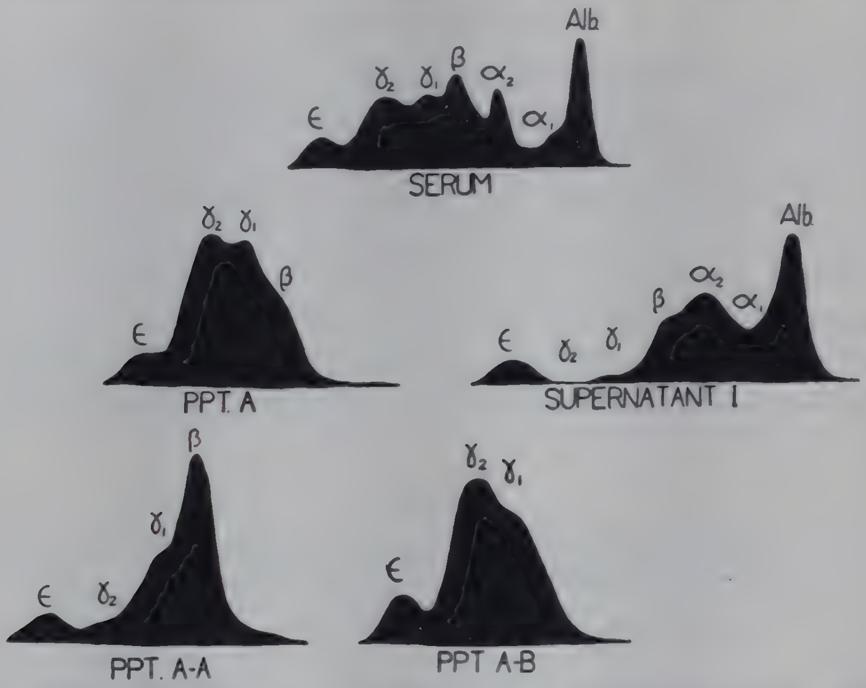


FIG. 1. Descending electrophoretic patterns of a hyperimmunized horse serum and fractions.

TABLE I

Antibody and Protein Recoveries from Serum of Hyperimmunized Horses

Antisera to	Units of antibody per 100 ml. plasma*	Weight of ppt. per 100 ml. serum			Units of antibody recovered per 100 ml. serum		
		Ppt. A	Supernatant to Ppt. A	Ppt. A-B	Ppt. A	Supernatant to Ppt. A	Ppt. A-B
Tetanus toxoid	20,000	5.07		3.22			24,200
Diphtheria toxoid	45,000	5.14		3.35			46,900
" "	129,000	5.24	3.17	3.51	98,000	3170	70,200
" "	65,000	5.55	3.36	3.47	55,500	2520	36,800
" "	57,500	5.21			52,100		
Tetanus toxoid	45,000	4.10			24,600		

* Plasma diluted with anticoagulant.

Refractionation of this precipitate to give Precipitate A-B may be accompanied by excellent yields in some cases and by considerable losses of antibody in others. It appears again that losses of antibody at this point may be due to causes just mentioned. Approximately 10 gm. of protein for every 50 gm. of Precipitate A are lost in preparing Precipitates A-A and A-B under the conditions employed for their separation. It can be seen from Table I that the major portions of the antibody are recovered in Precipitate A or in its subfraction Precipitate A-B. Approximately 2 to 5 per cent of the initial serum antibody may be recovered by lyophilization of Supernatant I. The yield of antibody into Precipitate A-A is relatively low. In a typical experiment, Precipitate A-A showed 100 units of tetanus antitoxin per gm. while Precipitate A-B gave 8000 units per gm. Subfractionations of Precipitate A-A give products with very low antibody content. They are composed largely of proteins moving with an electrophoretic mobility of -4.0×10^{-5} sq. cm. per volt per second (pH 8.6). Such findings indicate that the antitetanus activity of horse serum proteins does not ordinarily extend into the electrophoretic region of the horse serum designated as β-globulin in Fig. 1.

DIAGRAM 2

50 gm. Ppt. A, suspended in 4000 ml. H_2O; 0.05 M HAc added to pH 5.38; diluted to 5000 ml.; μ = 0.0014; centrifuged at 0°

7.8 gm. (dry) Ppt. A-A

Supernatant IIA, volume 4910 ml.; 150 ml. 0.15 M NaCl and 8 ml. 0.5 M $NaHCO_3$ added to pH 5.83; 1267 ml. 50% EtOH added to 10% ethanol concentration; volume 5068 ml.; μ = 0.0061; centrifuged at −2°

15.9 gm. (dry) Ppt. A-1B

Supernatant IIB, volume 4950 ml.; 1650 ml. 50% EtOH added to 20% ethanol concentration (pH 5.83); μ = 0.0046; volume 6600 ml.; centrifuged at −6°

8.5 gm. (dry) Ppt. A-2B

Supernatant IIC, volume 6482; 100 ml. 0.5 M $NaHCO_3$ added to pH 7.4; 500 ml. 95% EtOH added to 25% ethanol concentration; volume 7082, μ = 0.01; centrifuged at −7°

9.9 gm. (dry) Ppt. A-3B

Supernatant IID, discarded

The sera of the two horses immunized to a series of antigens were pooled and fractionated to yield the usual initial antibody-rich Precipitate A. These precipitates were subfractionated and the distribution of the antibodies in the various fractions was studied. The subfractionation conditions are shown in Diagram 2.

The predominant feature of this subfractionation was the separation of the usual Precipitate A-B into three fractions, a γ_1-globulin, a γ_2-globulin, and a mixture of these two globulins.

In Fig. 2 are shown the electrophoretic compositions of these subfractions. It is readily apparent that the component labeled γ_1-globulin is relatively heterogeneous electrically and the fraction as a whole has a considerably higher electrophoretic mobility than does the γ_2-fraction.

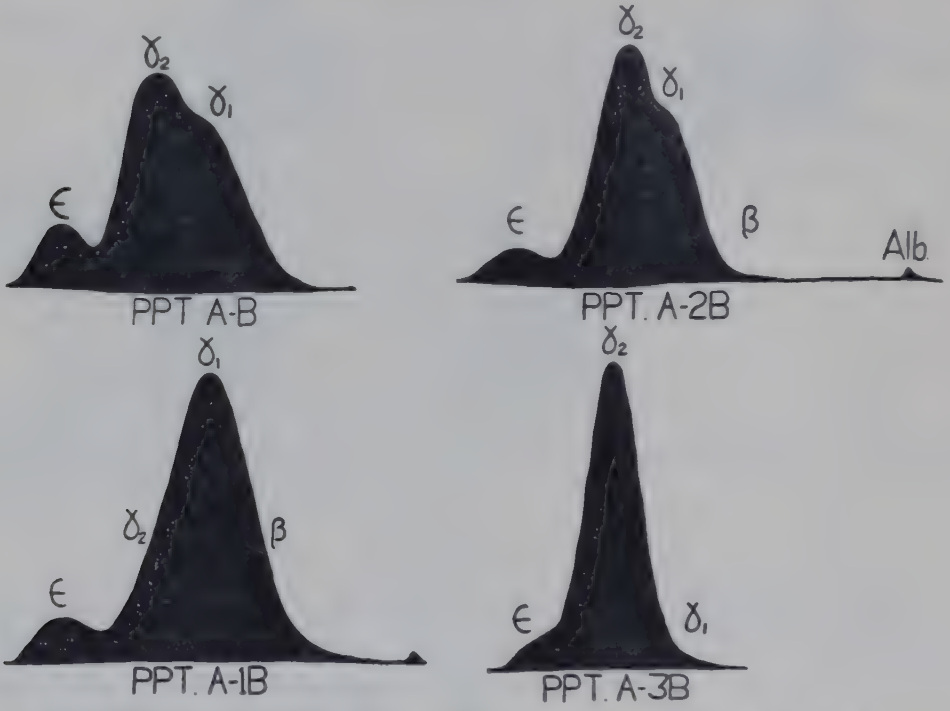


FIG. 2. Descending electrophoretic patterns of Precipitate A-B of a hyperimmunized horse serum and its subfractions.

Antibody and protein yield data are shown in Table II. As was anticipated, no antibodies to pneumococcus type III organisms were found in Precipitate A as tested by agglutination, capsular swelling phenomena, or by precipitin reactions with pneumococcus type III polysaccharide. The horses evidently failed to produce antibodies to the formalized pneumococcus vaccine which was employed. Antibody production to vibrion septique toxoid was relatively low (less than 200 units per gm. of Precipitate A) and the subfractions were not assayed further. The remaining antibodies were present in relatively low titer but were of sufficient magnitude to make possible a study of their distribution into the various fractions. Practically no antibody was found in the supernatant to Precipitate A. The subfractions of Precipitate A showed varying titers of the several antibodies studied. The initial precipitate removed at pH 5.2 and low ionic strength (Precipitate A-A) contained very small amounts of antitoxin but contained as much, or more, agglutinin for *Hemophilus pertussis* on a weight basis as did the parent fraction (Precipitate A). Approximately 10 to 15 per cent of the diphtheria and tetanus antitoxins was found in Precipitate A-1B with the remainder appearing in the more soluble protein (pseudoglobulin in nature) making up Precipitates A-2B and A-3B. The diphtheria antitoxin showed a relatively higher titer in the γ_2-globulin fraction (Precipitate A-3B) than was true for tetanus antitoxin. The antitoxin to *Bacillus welchii* was found to be rather well distributed in all of these fractions. The *Hemophilus pertussis* agglutinins showed a high concentration in the γ_2-globulin fraction as compared to the γ_1 and the $\gamma_1+\gamma_2$-globulin subfractions. Such a finding might have been predicted from the electrophoretic studies of van der Scheer *et al.* (5). The high titer of this antibody in the euglobulin type of precipitate (Precipitate A-A) is, however, quite surprising in view of the small amount of γ_2-globulin in this fraction and is in contrast to the antitoxin distribution in the same fraction.

TABLE II

Antibody Content of Subfractions of Antibody Fraction of Hyperimmune Horse Serum

Weight of fraction	Fraction	Units antitoxin per gm. protein			*Hemophilus pertussis* agglutinin titer
		Diphtheria	Tetanus	*Bacillus welchii*	
gm.					
50	A*	3000	1000	300	1-1280
7.8	A-A	300	100	−25	1-2560
15.6	A-1B (γ_1)	1000	500	300	1-160
8.4	A-2B ($\gamma_1 + \gamma_2$)	5000	4000	400	1-160
9.8	A-3B (γ_2)	3000	1000	500	1-1280

* The supernatant to Precipitate A contained less than 2 per cent of the antibody activity of this fraction.

Quantitative Diphtheria Antitoxin Assays—A γ_1-globulin, a γ_2-globulin, and a mixture of them (analogous in electrophoretic composition to Precipitates A-1B, A-3B, and A-2B respectively of Fig. 2) were prepared from pooled antidiphtheritic horse serum that assayed approximately 800 units per ml. The γ_1-globulin preparation, however, was separated from a $\gamma_1+\gamma_2$-globulin mixture (Precipitate A-2B), since this fraction has a relatively higher antidiphtheritic titer in contrast to Precipitate A-1B (see

Table II). No attempt was made to recover large amounts of antibody, attention being focused on the recovery of electrophoretically well defined fractions. The $\gamma_1+\gamma_2$-globulin fraction contained approximately equal amounts of the two component proteins. The antibody contents of these preparations were determined by the quantitative methods as elaborated by Heidelberger and associates (16).[4] These results, summarized in Table III, show a diphtheritic antitoxin distribution in the various fractions that was analogous to that found by *in vivo* assay in similar fractions of the polyvalent horse serum (see Table II). The shape of the quantitative precipitin curves for these fractions was essentially the same as that obtained by Kabat (17) in plotting the Pappenheimer and Robinson (18) data for the diphtheria toxin-antitoxin (horse) reaction. In agreement with results of Kekwick and Record (3) the γ_2-globulins were found to flocculate more readily with toxin than the γ_1-globulins.

TABLE III

Diphtheria Antitoxin Content of Various γ-Globulin Fractions by Quantitative Precipitin Methods

Fraction	Units of diphtheria antitoxin per gm. protein	Per cent antitoxin of fraction
γ_1-Globulins	9000	10.4
$\gamma_1+\gamma_2$-Globulins	6750	7.8
γ_2-Globulins	3070	3.6

Fractionation of Serum during Course of Immunization—Changes in the plasma proteins from a single horse were studied during the course of immunization to diphtheria toxoid. Serum samples were collected before and at various times during the immunization period. Unfortunately the animal employed did not develop antitoxin above 400 units per ml. and the experiment was discontinued at this point. The serum fractions obtained do, however, show the shift toward the development of large amounts of γ_1-globulin, as was expected from the previous serum electrophoretic studies of van der Scheer *et al.* (5, 6) and Kekwick and Record (3). Electrophoretic patterns and yields of some of the serum samples fractionated are shown in Fig. 3. A marked increase in the yield of Precipitate A-B as immunization continued is readily apparent. The predominating feature is the gradual increase of the γ_1-globulin. The effect is most readily observed as the electrophoretic diagrams for Precipitates A-3B are studied. As the γ_1-globulin content of the serum increases it likewise becomes more difficult to prepare a γ_2-globulin fraction (Precipitate A-3B) which does not show the presence of considerable γ_1-globulin. As previously noted by Kekwick and Record (3), there was an increased production of the γ_2-globulin during the initial stage of immunization. Thus, by the 7th day of immunization this component showed a marked enhancement without any increase in the amount of γ_1-globulin, but as immunization progressed the level of the γ_1-globulin rose.

---

[4] We wish to thank Mr. Melvin Cohn, Department of Microbiology, New York University, College of Medicine, for carrying out these determinations.

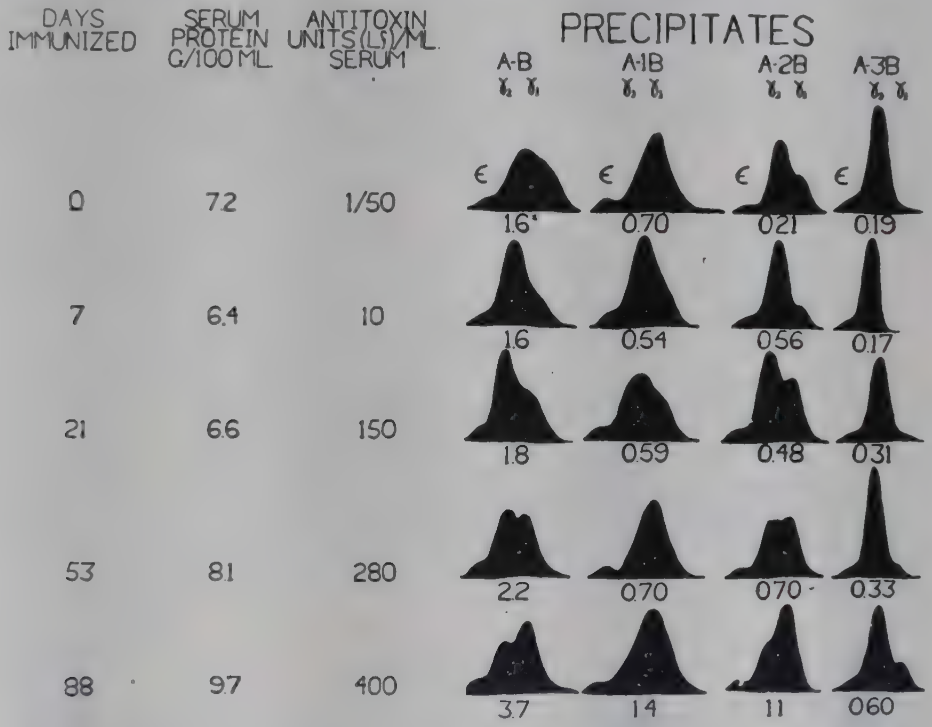


FIG. 3. Descending electrophoretic patterns and yield data of serum fractions from a single horse during immunization to diphtheria toxoid. The numbers under patterns are the yields of precipitate in gm. for 100 ml. of serum.

Fractionation of Normal Horse Sera—Serum samples which had been taken from nine normal horses were analyzed electrophoretically and fractionated individually in order to study the variation in composition and yield that one might expect when working with the plasma from single animals. The yield data of the fractions obtained from the sera examined are shown in Table IV. It is apparent that a great deal of individual variation may be expected among so called "normal" animals. Another feature of these and previous experiments was the increased difficulty in removing the β-globulins associated with Precipitate A into Precipitate A-A. This is in rather marked contrast with the results obtained when serum from hyperimmunized animals is used.

Electrophoretic Mobility Studies—The electrophoretic mobilities of γ_2-

and γ_1-globulin preparations obtained from antidiphtheritic plasma were determined for a series of pH values. The γ_1-globulin fraction represents that portion of Precipitate A from an antidiphtheritic serum which was soluble at pH 5.2, ethanol 10 per cent and ionic strength of 0.002, but which was insoluble at pH 6.1. This globulin preparation is somewhat analogous to Precipitate A-1B of Fig. 2, except that it was far more homogeneous electrically than the usual Precipitate A-1B. The γ_2-globulin was a fraction analogous to Precipitate A-3B of Fig. 2. The electrophoretic patterns of these fractions in pH 8.6 veronal buffer of ionic strength 0.1 are shown as γ_1- and γ_2-globulins in Fig. 4. It can be seen that the γ_1-globulin has a slightly asymmetric pattern. The marked electrical inhomogeneity in this fraction has been already mentioned and, even though the γ_2-globulin preparation appeared to be more homogeneous from its electrophoretic diagram, the heterogeneity constant (19) of various preparations gave values in the neighborhood of 1×10^{-5} sq. cm. per volt per second.[5] The

TABLE IV

Fractionation Results for Normal Horse Serum

Material		Per cent electrophoretic composition						Protein, gm. per 100 ml. starting serum
		γ_2	γ_1	β	α_2	α_1	Albumin	
Serum	Range	18–31	9–17	11–24	10–22	7–13	21–29	6.4–8.7
	Average	22	14	17	14	9	24	7.6
Ppt. A	Range	29–46	25–31	15–35	4–11	0–3	1–4	2.7–5.6
	Average	40	28	22	8	1	2	4.1
" A-A	Range	6–9	23–36	40–57	11–22	0–5	0–2	0.2–1.1
	Average	8	28	46	12	3	1	0.7
" A-B	Range	53–73	21–32	7–15	0–3	0	0–1	1.3–3.5
	Average	60	25	12	1	0	1	2.5

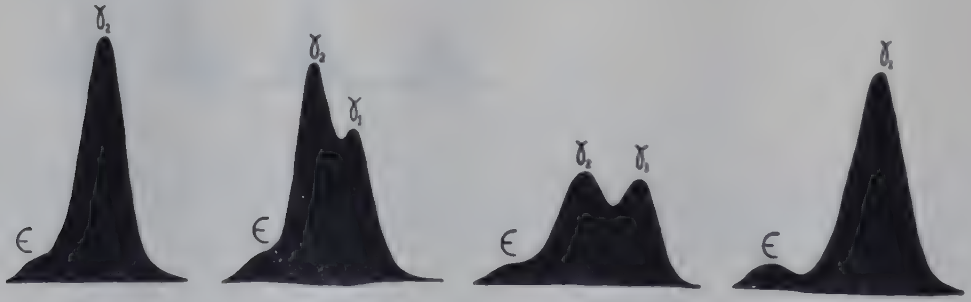


FIG. 4. Descending electrophoretic patterns of γ_1- and γ_2-globulins and mixtures of these two proteins.

---

[5] Anderson, E. A., and Nichol, J. C., unpublished experiments.

mobility *versus* pH values for the two protein fractions are plotted in Fig. 5. The average isoelectric points of the γ_1- and the γ_2-globulin components in buffer solutions of 0.1 ionic strength are 5.6 and 7.6 respectively. With the exception of the γ_1-globulin fraction at pH 8.6 these proteins gave single, apparently symmetrical peaks over the entire pH range studied.

Our investigations indicate that these globulin fractions are merely proteins of closely related isoelectric point and mobility. Dependent upon the conditions of separation there may be obtained arbitrary fractions which contain antibody and which have electrophoretic mobilities from -1.0 to -3.5×10^{-5} sq. cm. per volt per second in buffer of pH 8.6 and ionic strength 0.1. Hence the γ_1- and γ_2-globulin fractions employed for the determination of isoelectric point are merely two fractions, the molecules in each being more closely related electrically than are those of the parent fraction (Precipitate A-B, Fig. 1). In addition to the patterns of the relatively homogeneous fractions, Fig. 4 also shows the diagrams for two mixtures of γ_1- and γ_2-globulins. It is readily apparent that a series of protein fractions showing variations in the average net charge at a given pH may be separated from horse serum. This fact is substantiated by the electrophoretic diagrams and analytical data for the hyperimmune horse serum antibody fractions of Smith and Gerlough (8).

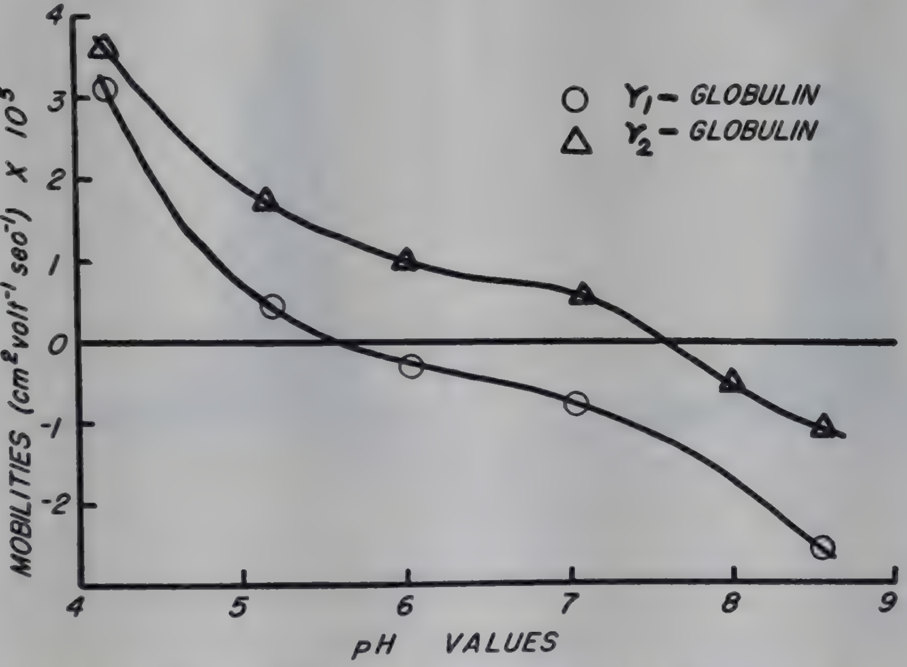


FIG. 5. pH mobility curve of a γ_1-globulin and a γ_2-globulin fraction

Sedimentation Studies—Various preparations of γ_2- and of γ_1-globulins and mixtures thereof were studied in the Svedberg high velocity oil turbine ultracentrifuge. Approximately 80 per cent of these globulin fractions consisted of material having a sedimentation constant (s_{20w}) of 6.8 or 7.2 Svedberg units, respectively. The remaining 20 per cent of the protein in each case sedimented with velocities between 8 and 15 Svedberg units and exhibited no well defined molecular components in this range. Some schlieren patterns of these proteins obtained during velocity sedimentation experiments are shown in Fig. 6. The main component of both of these fractions was molecularly monodisperse, showing no increase in the apparent diffusion constant as sedimentation progressed. A mixture of the γ_1- and γ_2-globulins exhibited the molecular mass behavior to be expected of a system of this kind.

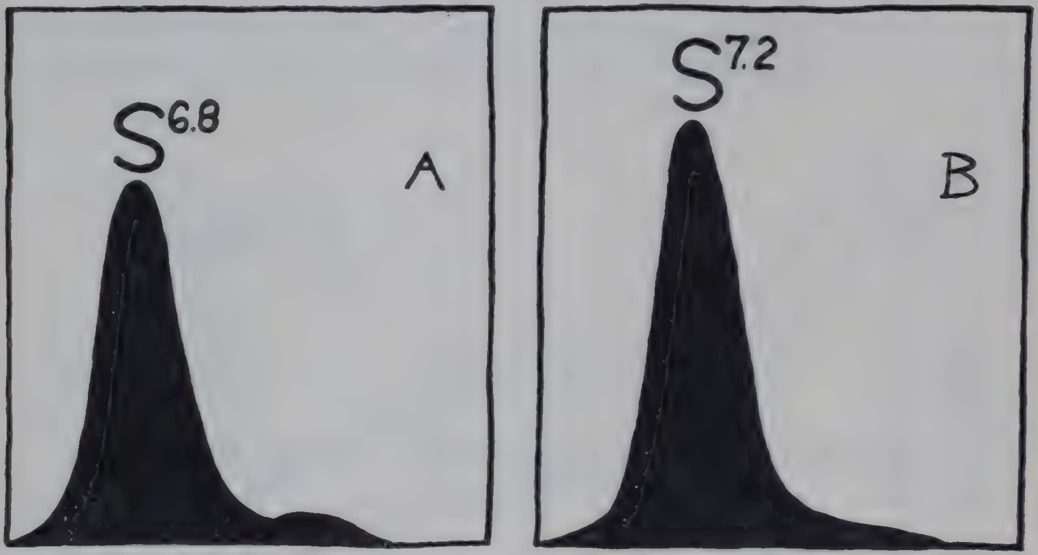


FIG. 6. Sedimentation patterns after 72 minutes at 220,000 times gravity of (*A*) γ_2-globulin fraction, (*B*) γ_1-globulin fraction.

DISCUSSION

The proteins of horse serum which are concerned with antibody activity can be readily separated from the serum by means of ethanol fractionation. In this respect the γ-globulins from hyperimmune sera are more easily separated free of β-globulin than are those from normal sera. The antibody fractions are relatively heterogeneous electrophoretically and may be separated into a series of fractions, the major components of which may show electrophoretic mobilities anywhere from -1.0 to -3.5×10^{-5} sq. cm. per volt per second in veronal buffer at pH 8.6 and ionic strength 0.1. Since these protein fractions show antibody activity, we have called them all γ-globulins in preference to using a series of unrelated terms such as γ, β_2, and T component (8), or γ, β_1, and β_2 (3), as has been done previously. The findings of Kekwick and Record are in agreement with the results of our work insofar as they may be compared.

The antibodies to the antigens we have studied appear to be distributed electrophoretically throughout this γ-globulin region. The γ_1- and γ_2-globulin fractions both contain antibody to a given antigen. The marked concentration of antibodies which make up only a small per cent of the total protein is not as readily achievable as it would be if the antibody were contained in a small and electrophoretically distinct component. The antibodies in question possess the same solubilities as do the γ_1- and γ_2-globulin serum components and, in order quantitatively to remove serum antibodies, these proteins must be likewise separated. Hence in preparing antibody-rich precipitates from horse serum the investigator is limited by the amount of antibody per unit of starting serum. Some slight difference in the solubilities of a given antibody is indicated by the results of the subfractionations of Precipitate A-B, as shown in Table II.

Fractionation of such protein systems must take into account isoelectric point and solubility distributions. For the antitoxins it would appear that, in agreement with previous findings, these antibodies are water-soluble and that furthermore they seem to be associated largely with the γ_1-globulins. Thus the γ_2-globulin fraction (Precipitate A-3B, Fig. 4) is much lower in antitoxin content than the water-soluble portion of the γ_1-globulin (see Table III). Thus Precipitate A-1B of Table II contains far more γ_1-globulin than does Precipitate A-2B but the antidiphtheritic potency of the latter fraction is far greater. This is apparently due to the γ_1-globulin of Precipitate A-2B being largely pseudoglobulin in nature as contrasted to the euglobulin characteristics of the globulin in Precipitate A-1B. As a further consequence of these fractionation conditions, the γ_2-globulins of Precipitate A-3B would tend to be more pseudoglobulin in nature than the analogous component in Precipitate A-2B. Such evidence is further indication that the antitoxic globulins of horse serum are more highly concentrated in the γ-globulins of lower isoelectric points and, as known before (20, 21), in the pseudoglobulin portions.

The bacterial antibodies as exemplified by the *pertussis* agglutinins show quite another behavior. Van der Scheer *et al.* (5) have indicated that bacterial antibodies appear to follow the γ_2-globulin component rather than the γ_1-component. The findings of Tiselius and Kabat (1), however, have indicated that horse pneumococcus antibody was a constituent corresponding to the γ_1-globulin (T component). Unfortunately, the two horses immunized in our work did not produce antibodies to the pneumococcus vaccine employed. It is difficult to reconcile the high content of *pertussis* agglutinin in both Precipitates A-A and A-3B (Table II). The former fraction is quite low in γ_1-globulins and very low in γ_2-globulins, while the Precipitate A-3B fraction is essentially all γ_2-globulin. The small amount of γ_1- and γ_2-globulin found by electrophoretic analysis in Precipitate A-A and the low content of antitoxin are consistent. The euglobulin nature of Precipitate A-A is in agreement with the presence of the anticarbohydrate and certain antiprotein globulins of horse serum in the water-insoluble fractions (22–24). However, Precipitate A-3B is essentially water-soluble and likewise shows a large amount of *pertussis* agglutinin.

A further factor that must be considered is the degree of immunization that a particular animal has undergone. It must be realized, too, that both the chemical nature of the antigen and its route of administration are important factors in determining the characteristics of the antibody formed (24). The data of Fig. 3 clearly indicate a great deal of variation in the fractions obtained under analogous conditions from a single horse serum as the period of immunization progresses. Since the antibody fractions of the horse appear to be so complex, it would appear that a great deal of information would be gained by a careful and extended study of the physiology and rate of production of the serum proteins.

The horse γ-globulins separated in this work have sedimentation constants (s_{20w}) in the neighborhood of 7 Svedberg units. This figure is in agreement with previous data (2, 3, 25, 26) for horse globulin. No component having $s_{20w} = 18$ Svedberg units was observed to be present in our fractions. Small amounts of such heavy protein material have been found in the sera of apparently normal horses (25). The horse pneumococcus antibody (27, 28) is known to possess a sedimentation constant $s_{20w} = 18$ Svedberg units. The γ_2-globulin fractions gave values which were consistently slightly less than $s_{20w} = 7$ Svedberg units, while the γ_1-globulin fractions always gave a somewhat higher figure. Approximately 20 per cent of the protein sedimented at a rate corresponding to $s_{20w} = 8$ to 15 Svedberg units as a relatively polydisperse mixture. The γ_2-globulins from other animal plasmas also have been found to separate with varying amounts of this faster sedimenting material (15, 29, 30). It is not known whether such protein results from the fractionation conditions used or whether the γ-globulins exist as such in nature.

While the so called γ_1- and γ_2-globulin fractions were definitely heterogeneous on electrophoresis at pH 8.6, the peaks showed no tendency to give more than one main component over a wide pH range. As separated, the γ_1-globulin fractions were far more heterogeneous in nature than the γ_2-globulin fractions. However, the relatively homogeneous γ_2-globulin fraction obtained represents only a small portion of the serum proteins usually designated as γ_2-globulin (Fig. 1).

The ability of the β-globulins to separate more readily from hyperimmune sera is probably related to increased concentration of the γ-globulins. Thus while relatively constant amounts of β-globulin are precipitated in all sera, they represent a smaller per cent of the total globulin precipitated from the immune systems. An evaluation of the amount of β-globulin in the serum or in an antibody fraction is difficult for, as seen from Fig. 1, it does not resolve well from the area described in an electrophoretic diagram as being due to γ_1-globulin. The very low content of antibody in such fractions as Precipitate A-A (Fig. 1) strongly suggests that antibody activity is not associated with β-globulin.

SUMMARY

An antibody-rich protein fraction of hyperimmunized horse plasma which is made up largely of γ_2- and γ_1-globulins may be readily separated from serum by ethanol fractionation. The antibodies appear to be distributed among molecules showing a wide variation in electrophoretic mobility. The largest amounts of antitoxin appear to be associated with the water-soluble portions of the γ_1-globulin fractions which is in contrast to the *pertussis* agglutinin.

The authors are extremely grateful to Eli Lilly and Company for their generous aid and assistance in carrying out certain phases of this work. They likewise wish to acknowledge the interest of Dr. J. W. Williams during the course of this investigation.

BIBLIOGRAPHY

1. Tiselius, A., and Kabat, E. A., *J. Exp. Med.*, **69,** 119 (1939).
2. Pappenheimer, A. M., Jr., Lundgren, H. P., and Williams, J. W., *J. Exp. Med.*, **71,** 247 (1940).
3. Kekwick, R. A., and Record, B. R., *Brit. J. Exp. Path.*, **22,** 29 (1941).
4. van der Scheer, J., and Wyckoff, R. W. G., *Proc. Soc. Exp. Biol. and Med.*, **43,** 427 (1940).
5. van der Scheer, J., Wyckoff, R. W. G., and Clarke, F. H., *J. Immunol.*, **39,** 65 (1940).
6. van der Scheer, J., Wyckoff, R. W. G., and Clarke, F. H., *J. Immunol.*, **40,** 173 (1941).
7. Deutsch, H. F., Alberty, R. A., and Gosting, L. J., *J. Biol. Chem.*, **165,** 21 (1946).
8. Smith, E. L., and Gerlough, T. D., *J. Biol. Chem.*, **167,** 679 (1947).
9. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, **68,** 459 (1946).
10. Parfentjen, I. A., U. S. patents 2,065,196 and 2,123,198 (1936).
11. Pope, C. G., *Brit. J. Exp. Path.*, **20,** 201 (1939).
12. Gjessing, E. C., Ludewig, S., and Chanutin, A., *J. Biol. Chem.*, **170,** 551 (1947).
13. Gjessing, E. C., and Chanutin, A., *J. Biol. Chem.*, **169,** 657 (1947).
14. Nichol, J. C., and Deutsch, H. F., *J. Am. Chem. Soc.*, **70,** 80 (1948).
15. Hess, E. L., and Deutsch, H. F., *J. Am. Chem. Soc.*, **70,** 84 (1948).
16. Heidelberger, M., *Bact. Rev.*, **3,** 49 (1939).
17. Kabat, E. A., *J. Immunol.*, **47,** 513 (1943).
18. Pappenheimer, A. M., Jr., and Robinson, E. S., *J. Immunol.*, **32,** 291 (1937).
19. Alberty, R. A., *J. Am. Chem. Soc.*, **70,** 1675 (1948).

20. Hiss, P. H., and Atkinson, J. P., *J. Exp. Med.*, **5**, 47 (1901).
21. Ledingham, J. C. G., *J. Hyg.*, **7**, 65 (1907).
22. Heidelberger, M., *J. Exp. Med.*, **86**, 77 (1947).
23. Treffers, H. P., Heidelberger, M., and Freund, J., *J. Exp. Med.*, **86**, 83 (1947).
24. Treffers, H. P., Heidelberger, M., and Freund, J., *J. Exp. Med.*, **86**, 95 (1947).
25. Svedberg, T., and Pedersen, K. O., The ultracentrifuge, Oxford (1940).
26. McFarlane, A. S., *Biochem. J.*, **29**, 660 (1935).
27. Heidelberger, M., Pedersen, K. O., and Tiselius, A., *Nature*, **138**, 165 (1936).
28. Heidelberger, M., and Pedersen, K. O., *J. Exp. Med.*, **65**, 393 (1937).
29. Williams, J. W., Petermann, M. L., Colovos, G. C., Goodloe, M. B., Oncley, J. L. and Armstrong, S. H., Jr., *J. Clin. Invest.*, **23**, 433 (1944).
30. Smith, E. L., *J. Biol. Chem.*, **165**, 665 (1946).

THE FATTY ACIDS OF CHLORELLA

By HAROLD W. MILNER

(*From the Carnegie Institution of Washington, Division of Plant Biology, Stanford, California*)

(Received for publication, July 19, 1948)

It has been found that the chemical composition of *Chlorella pyrenoidosa* varies over a wide range, depending on the environmental conditions selected for its growth. A description of the influence of different environmental factors on the composition of *Chlorella* has been given, and a method of calculating the approximate cell composition in terms of carbohydrate, protein, and lipide from the elementary analysis was developed (1). The most striking variation occurred in the calculated lipide content of the cells, which ranged from 5 to 85 per cent of the dry weight.

Little, if any, information is available on the composition of the fats of algae. The purpose of the work to be described here was to determine by analysis the lipide content of *Chlorella* grown under various conditions, to determine the composition of the lipide fraction, and, more particularly, to examine the fatty acids of *Chlorella*.

EXPERIMENTAL

Plant Material

The production of *Chlorella* having either extremely low or extremely high lipide content requires a much longer time and more specialized experimental conditions than those required to produce cells having between 20 and 75 per cent lipide. In order to obtain sufficient material for analysis of the lipide fraction of *Chlorella* cells having different lipide contents, large scale culture was undertaken to produce cells having about 20, 35, 60, and 75 per cent lipide.

Lot 1—A composite lot of cells was obtained from cultures grown in 5 gallon bottles in a greenhouse, as described elsewhere (2). The mineral nutrient medium contained 0.020 M $MgSO_4$, 0.018 M KH_2PO_4, 0.025 M KNO_3, and 0.000005 M $FeSO_4$ dissolved in previously boiled, cooled, and filtered tap water. The cultures grew for 80 days under natural illumination and were aerated with 5 per cent CO_2 in air. 1 kilo, dry weight, the yield from about 30 cultures, was used for lipide analysis.

Lot 2—Seven cultures, 15 liters each, in 5 gallon bottles were grown outdoors, near a north wall, for 17 days. The medium contained 0.010 M $MgSO_4$, 0.010 M KH_2PO_4, 0.000825 M $NH_4H_2PO_4$, 0.000715 M $(NH_4)_2HPO_4$, 0.030 M KCl, and 0.000005 M $FeCl_3$ dissolved in water pretreated as for Lot

1. 5 per cent CO_2 in air was bubbled through the cultures. The yield from seven cultures was 66.5 gm. dry weight.

Lot 3—The medium and conditions of growth were the same as for Lot 2, except that the time of growth was 83 days. The yield was 113 gm. dry weight from six 15 liter cultures.

Lot 4—Conditions known to produce cells of very high lipide content were employed in growing this lot of *Chlorella*. The medium was the same as in Lots 2 and 3. The gas stream was 5 per cent CO_2 in nitrogen. Ten cultures, each 2 liters in volume, were grown in Fernbach flasks. Each culture was illuminated continuously for 75 days by a 200 watt Mazda lamp 15 cm. below the flask. A water bath kept the temperature of the culture at 21–23°. The ten cultures yielded 51.6 gm. dry weight.

TABLE I

Composition of Chlorella

Lot No.	Found by analysis				Calculated		
	C	H	N	Ash	Carbohydrate	Protein	Lipide
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	48.64	6.88	6.80	7.71	33.4	46.4	20.2
2	53.46	7.66	4.15	4.97	37.5	27.3	35.2
3	60.95	8.80	2.01	4.69	23.5	13.1	63.4
4	65.58	9.43	1.23	3.49	15.0	7.9	77.1

Each culture was examined microscopically before the *Chlorella* cells were collected. No organism other than *Chlorella* was present in any of the cultures used in this work.

In order to show the difference in elementary composition of *Chlorella* of various lipide contents, and in order to check the validity of calculating the approximate carbohydrate, protein, and lipide content from the elementary analysis (1), these data for the four lots of *Chlorella* described above are shown in Table I.

Analytical Methods

Lipides—The extraction of lipides from dry *Chlorella* by anhydrous fat solvents is slow and usually incomplete. The extraction becomes quantitative and reasonably rapid if the dry plant material is first treated with water and then dehydrated with methanol. In this work, the dry, pulverized *Chlorella* was thoroughly mixed with twice its weight of water; then methanol was added slowly with continuous stirring until the alcohol concentration was 95 per cent by volume. The mixture was boiled 1 hour under a reflux, then filtered. The material was next extracted three times under a reflux with anhydrous methanol. Extraction was continued, methanol and petroleum ether, b.p. below 70°, being used alternately until the material and extracts became colorless. A final extraction was made with ether. Usually no more than a trace of lipide was found in this last extract. The combined crude lipides from all the extractions were freed of solvent *in vacuo*. In order to remove the non-lipide materials which had been extracted by methanol, the crude lipide was taken up in anhydrous, alcohol-free ether and the solution filtered. Some of the non-lipide ether-insoluble material which was separated in this step was crystalline. The crystals were identified as sucrose. The ether was removed from the filtered lipide solution at reduced pressure and the lipide was dried to constant weight *in vacuo*. This material is reported as total lipide.

Because of the easily oxidizable nature of the extracted material, extracts were exposed to air as little as possible and were kept *in vacuo* between operations. All solvents were freshly redistilled just before use.

The total lipide from each lot of *Chlorella* was saponified by boiling 3 hours under a reflux with 5 per cent KOH in methanol, 10 ml. per gm. of lipide being used. Most of the methanol was then removed by distillation. The solution was diluted with water to its original volume. Unsaponifiable material was removed by thorough extraction with ether and its weight was determined after freeing it of solvent. The fatty acids were liberated by acidification of the aqueous solution with H_2SO_4 and were transferred quantitatively into low boiling petroleum ether. The solvent was removed at reduced pressure and the fatty acids were dried *in vacuo*.

Fatty Acids—The fractionation of the fatty acids was accomplished by the usual methods of lead salt separation and by distillation of the methyl esters. The total fatty acids from Lots 1 and 3 were first converted into methyl esters. The esters were fractionally distilled through a 12 inch Widmer column at 1 mm. pressure. Only a trace of ester distilled at a lower temperature than the boiling point of esters of the C_{16} acids. A sharp separation was obtained between esters of C_{16} and C_{18} acids. After distillation of the esters of C_{18} acids was complete, a residue amounting to 5 per cent of Lot 1 and 2 per cent of Lot 3 remained undistilled. Efforts to fractionate these residues were unsuccessful. They may have contained a small amount of esters of C_{20} or higher acids, plus decomposition products. The distilled esters were reconverted into the free acids, which were separated by the lead salt method into saturated and unsaturated fractions.

Lots 2 and 4 of *Chlorella* yielded an insufficient quantity of fatty acids to permit accurate separation of the methyl esters in the apparatus used for the larger lots. The acids from Lots 2 and 4 were not esterified, but were separated directly into saturated and unsaturated fractions by the lead salt method.

The equivalent weight and iodine number (Hanus) of each lot of total fatty acids, and of each of the fractions of these, were determined. From these values the composition of the fatty acid mixture from each lot of *Chlorella* was calculated. The percentage of acids other than those of the C_{16} and C_{18} series was too small to be determined in the amount of material available and by the methods used. For that reason, the sum of C_{16} and C_{18} fatty acids in *Chlorella* is here taken to be 100 per cent.

TABLE II

Analysis of Lipide Fraction of Chlorella

Line No.	Analysis	*Chlorella* lot No.			
		1	2	3	4
1	Total lipide, *% of Chlorella*	23.37	33.17	62.96	75.51
	Composition of total lipide				
2	Fatty acids, *% of lipide*	28.0	49.5	83.0	86.8
3	Unsaponifiable, *% of lipide*	12.0	7.7	3.3	3.3
4	Water-soluble saponification products, *% of lipide*	60.0	42.8	13.7	9.9
5	Calculated fat, *% of Chlorella*	6.85	17.2	54.7	68.6
	Analysis of total fatty acids				
6	Iodine No. (Hanus)	163.1	143.8	143.6	125.3
7	Equivalent weight	269.5	273.6	272.7	274.1
8	Palmitic acid, *% of total*	16.6	10.9	7.9	11.4
9	Stearic acid, % " "	0.4	4.1	3.9	3.5
10	C_{16} unsaturated, *% of total*	29.1	18.3	27.3	18.0
11	C_{18} " % " "	53.9	66.7	60.9	67.1
	Degree of unsaturation				
12	C_{16} unsaturated acids	−4.1 H		−4.4 H	
13	C_{18} " "	−4.5 "		−3.4 "	
14	C_{16} + C_{18} unsaturated acids		−3.6 H		−3.2 H

RESULTS AND DISCUSSION

The analytical data for the lipides from the four lots of *Chlorella* are summarized in Table II. Comparison of the calculated lipide content shown in Table I with that found by analysis, Line 1 in Table II, shows good agreement, considering that the calculations assume a fixed composition of the lipide fraction, whereas analysis demonstrates that it varies between one lot of *Chlorella* and another.

Analysis of the lipides, Lines 2, 3, and 4 of Table II, shows a marked increase in the fatty acid content as the total amount of lipide increases, accompanied by a corresponding decrease in the unsaponifiable fraction. The increase in lipide content of *Chlorella* is mainly due to the accumulation of fatty acids. No significant accumulation of hydrocarbons can have occurred.

It would have been of interest to determine the nature of the considerable quantity of water-soluble saponification products, Line 4, which were discarded. Chlorophyll degradation products could account for about half of this fraction in Lots 1 and 2. The chlorophyll content of *Chlorella* decreases rapidly as the lipide content increases. Lot 1 of *Chlorella* had 6 per cent dry weight of chlorophyll, while Lot 4 had only 0.03 per cent. Glycerol would constitute part of the material included in Line 4, a substantial part in Lots 3 and 4.

For comparison with the total lipide contents, Line 1, the fat contents were computed. The total fatty acids of each lot were calculated to triglycerides and the latter expressed as percentages of the *Chlorella*, Line 5.

As the lipide content of *Chlorella* increases, there is a significant decrease in the degree of unsaturation of the fatty acids, Line 6. The average molecular weight of the fatty acids, Line 7, is almost equal in Lots 2, 3, and 4, and is slightly lower in Lot 1. With the exception of the acids from Lot 1, the percentage of the different fractions, Lines 8 to 11, does not show a clearly defined relation to the change in fatty acid content of the *Chlorella*. Most of the saturated fatty acid is palmitic, with only a small amount of stearic acid.

The remarkable feature of the fatty acids from *Chlorella* is the great unsaturation of the liquid acids, Lines 12 to 14, particularly of the C_{16} fraction. The iodine number, 217.2, of the C_{16} unsaturated acids from Lot 3 requires the presence of at least 17 per cent of triply unsaturated acids in this fraction. Triply unsaturated C_{18} acid is required only in Lot 1 in order to account for the iodine number of that fraction. Comparison of the acids from Lots 1 and 3 shows that the over-all decrease in unsaturation can be attributed to the C_{18} acids, since the C_{16} acids of Lot 3 are even more unsaturated than those of Lot 1.

SUMMARY

Through control of environmental factors, four lots of *Chlorella pyrenoidosa* were grown, which contained 23, 33, 63, and 76 per cent lipide. The fatty acid content varied from 6 to 66 per cent of the dry weight of the cells. Analysis of the fatty acid mixtures showed that saturated acids, mostly palmitic, comprise 12 to 16 per cent of the total, and that the liquid acids are highly unsaturated.

BIBLIOGRAPHY

1. Spoehr, H. A., and Milner, H. W., *Plant Physiol.*, in press.
2. Spoehr, H. A., Smith, J. H. C., Strain, H. H., Milner, H. W., and Hardin, G. J., Fatty acid antibacterials from plants, Carnegie Institution Publication, in press.

METHIONINE IN SELENIUM POISONING*

BY I. GORDON FELS† AND VERNON H. CHELDELIN

(*From the Department of Chemistry, Oregon State College, Corvallis*)

(Received for publication, June 24, 1948)

The problem of selenium poisoning in farm animals has excited interest both from an academic and practical point of view. However, since the pioneering work of K. W. Franke and his coworkers, and the discovery of selenium in toxic grains by Robinson,[1] relatively less attention has been paid to the mode of action of selenium compounds or means of detoxification.

At an early date Hofmeister (1) reported that selenium salts were eliminated from the lungs in the form of volatile methylated derivatives. Inasmuch as the compounds were not isolated, this conclusion has been questioned (2, 3). The possibility that selenium may take the place of sulfur in the synthesis of mercapturic acids has been suggested by the observations of Moxon *et al.* (4). The feeding of bromobenzene to selenized steers resulted in a reduction of the selenium content of the blood with a corresponding rise in urinary selenium. Other workers (5), however, were not able to duplicate these findings in rabbits. The observation that arsenite is capable of alleviating selenium toxicity in animals is at present inexplicable (6).

The present communication deals primarily with the toxicity of selenate and its counteraction by methionine in yeast.

EXPERIMENTAL

The organism used was *Saccharomyces cerevisiae* (Fleischmann) which was transferred daily on molasses agar.[2] The medium used throughout the study was a modification of that used by Williams and Saunders (7) with

---

* This work was supported by a grant from Swift and Company, Inc.

Published with the approval of the Monographs Publications Committee, Oregon State College. Research Paper No. 127, School of Science, Department of Chemistry. Presented before the Division of Biological Chemistry, American Chemical Society, 113th meeting, Chicago, April, 1948. A preliminary report was given at the sixth meeting of the Oregon Academy of Science, Salem, February, 1948.

† Swift and Company Fellow in Biochemistry.

[1] For an excellent review on selenium poisoning the reader is referred to Painter's monograph (Painter, E. P., *Chem. Rev.*, **28**, 178 (1941)).

[2] The molasses agar has the following composition: 4 per cent molasses, 0.12 per cent $NH_4H_2PO_4$ and 2 per cent agar.

the same organism. Since preliminary experiments indicated that the extent of inhibition depended upon the amount of sulfate present (*cf.* analogy in plants (8, 9)), the sulfate content was reduced to 0.5 gm. of $Na_2SO_4 \cdot 10H_2O$ per liter of medium. This concentration permitted optimum growth of yeast in the absence of selenate, but was sufficiently low to exert no influence on the inhibition caused by 1 mg. of selenate per tube. The double strength medium is given in Table I, where the percentages in parentheses are those of sulfur present as contamination.

All tests were carried out in 20 × 150 mm. lipless Pyrex test-tubes, which received 5 ml. of the double strength medium, followed by any ingredients to be tested, and finally with water to a volume of 10 ml.

TABLE I

Basal Medium for Saccharomyces cerevisiae (Fleischmann)

Sucrose	40	gm.	(0.000 %)	$FeCl_3$	1.0	mg.
$(NH_4)_2$ tartrate	6	"	(0.001 %)	$CuCl_2$	0.2	"
KH_2PO_4	4	"	(0.000 %)	KI	0.2	"
Asparagine	3	"	(0.000 %)	Inositol	5	"
$Na_2SO_4 \cdot 10H_2O$	0.5	"		Thiamine hydrochloride	40	γ
$CaCl_2 \cdot 2H_2O$	0.5	"	(0.01 %)	β Alanine or calcium pantothenate	0.5	mg.
$MgCl_2$	0.5	"	(0.000 %)			
$TlCl_3$	2	mg.		Pyridoxine	40	γ
$ZnCl_2$	2	"		Biotin	1	"
$MnCl_2$	2	"		Distilled H_2O to	1000	ml.
H_3BO_3	2	"		pH	5.3	

The tubes were sterilized by steaming for 15 minutes, and inoculated when cool. It was found that the maintenance of sterility could be accomplished by covering the tubes with a sterile towel instead of the usual cotton plugs.

For an inoculum, four to five loopfuls of yeast from a freshly grown slant were homogeneously suspended in 15 ml. of sterile saline. This was centrifuged and resuspended in another 15 ml. of saline. The yeast concentration as determined turbidimetrically was approximately 0.8 mg. per ml. on a wet weight basis. 1 drop of this suspension was used as the inoculum, although it was found that the size of the inoculum was not critical; 5 drops yielded the same results as 1. The tubes were incubated at 30–31° for 2 to 3 days and the turbidity was measured in a photoelectric turbidimeter with a 5400 A filter. Growth was expressed in terms of optical density which is equal to log 100 minus per cent transmission (2 − log *G*).

Reversal of Selenate Toxicity by Methionine

Preliminary experiments showed that hydrolyzed casein was capable of reversing the inhibition produced by 1 mg. of selenate[3] per culture of yeast. By using the individual amino acids in 2 mg. quantities per tube, it was found that only methionine (the DL variety was employed) possessed such activity. Negative results were obtained with the following substances: L-tryptophan, DL-isoleucine, L-leucine,[4] DL-alanine, DL-phenylalanine, L-histidine, DL-valine, glycine, DL-glutamic acid, DL-serine, L-arginine, L-cystine, or 2 mg. of a mixture of these amino acids. Negative results were also obtained with 2 mg. of each of the following: L-cysteine,[5] glutathione,[5] DL-ethionine,[6] thymine, cytosine, guanosine, guanylic acid, adenosine, adenylic acid, 2-methyl-6-aminopyrimidine, 2-methyl-5-ethoxymethyl-6-aminopyrimidine, hydrolyzed desoxyribonucleic acid[7] as prepared by Levene and Bass (14), and 200 γ of each of the following: 2-amino-4-hydroxy-6-methylpyridine, 2-amino-4-hydroxypyridine-6-carboxylic acid, and pteroic acid.

The relationship of DL-methionine to selenate inhibition is depicted in Table II and shown graphically in Fig. 1. Straight lines are obtained when the plot is made on a logarithmic scale. Concentrations of methionine above 3 mg. per tube did not enhance the reversal.

In view of the fact that animals can utilize homocystine plus a methyl donor such as choline or betaine (15, 16) in lieu of methionine, an attempt was made to determine whether yeast can use these substances to reverse the effect of selenate. No activity was noted with 2 mg. of DL-homocystine, choline, betaine, or creatinine, either individually or in combination.

Action of Methionine-Free Casein Hydrolysate

The specificity of methionine reversal was also tested by noting the effect of a methionine-free casein hydrolysate upon selenate activity. The hydrolysate was prepared as follows, with a modification of Baernstein's method for determining methionine in protein hydrolysates (17): 500 mg. of Casamino acids (Difco) were refluxed with 25 ml. of freshly distilled HI (sp. gr. 1.7) for 8 hours in a CO_2 atmosphere. The excess HI was removed by vacuum distillation, a few drops of dilute HCl being used to facilitate removal. A silver chloride suspension (prepared by precipitating Ag_2O from $AgNO_3$ and NaOH, centrifuging, decanting the supernatant, and adding strong HCl to form AgCl) was added in excess to the hydrolysate. This was added to a large centrifuge tube, shaken for 10 minutes, centrifuged, and filtered. The precipitate was washed twice and the washings were added to the filtrate. The latter was evaporated to dryness and reconstituted to 50 ml. The final concentration was thus 10 mg. per ml. The filtrate gave a negative McCarthy-Sullivan test for methionine.

---

[3] The authors are indebted to E. P. Eddy and W. E. Caldwell for the gift of 87.2 per cent H_2SeO_4 prepared by the method of Gilbertson and King (10). In all these experiments the pH of all ingredients was adjusted to that of the medium before use.

[4] Partial activity was obtained with one sample of leucine (Eastman). However, a sodium fusion yielded a positive sulfur test, and the sample gave a positive test for methionine by the McCarthy-Sullivan method (11). Previous authors (12, 13) have warned of methionine contamination of leucine in isolation products. When synthetic leucine was used (Merck), no reversal was noted.

[5] L-Cysteine and glutathione reacted with H_2SeO_4 to form elemental Se. This took place even prior to the inoculation, but no reversal was evidenced.

[6] Kindly furnished by United States Industrial Chemicals, Inc.

[7] Obtained from the Krishell Laboratories, Portland, Oregon.

TABLE II

Relationship of DL-*Methionine to Selenate Inhibition in Saccharomyces cerevisiae*

Incubation time, 52 hours.

H_2SeO_4	Methionine	Optical density
mg.	*mg.*	
0	0	0.980
1	0.0	0.000
1	0.5	0.381
1	1.0	0.718
1	2.0	0.770
1	3.0	0.842
2	0	0.000
2	0.5	0.232
2	1.0	0.660
2	2.0	0.732
2	3.0	0.785
3	0	0.000
3	0.5	0.212
3	1.0	0.640
3	2.0	0.650
3	4.0	0.708
4	0	0.000
4	0.5	0.164
4	1.0	0.631
4	2.0	0.680
4	3.0	0.702
5	0	0.000
5	0.5	0.125
5	2.0	0.675

The effects of this hydrolysate, with and without supplementation with DL-methionine, are summarized in Table III. No reversal of selenate inhibition was obtained with this preparation, but when DL-methionine was also added the growth of yeast seemed fully restored. Although the levels employed do not permit a precise calculation of the reversing power of ordinary casein hydrolysate in terms of methionine content, it is evident that the casein effect is due principally, if not entirely, to methionine.

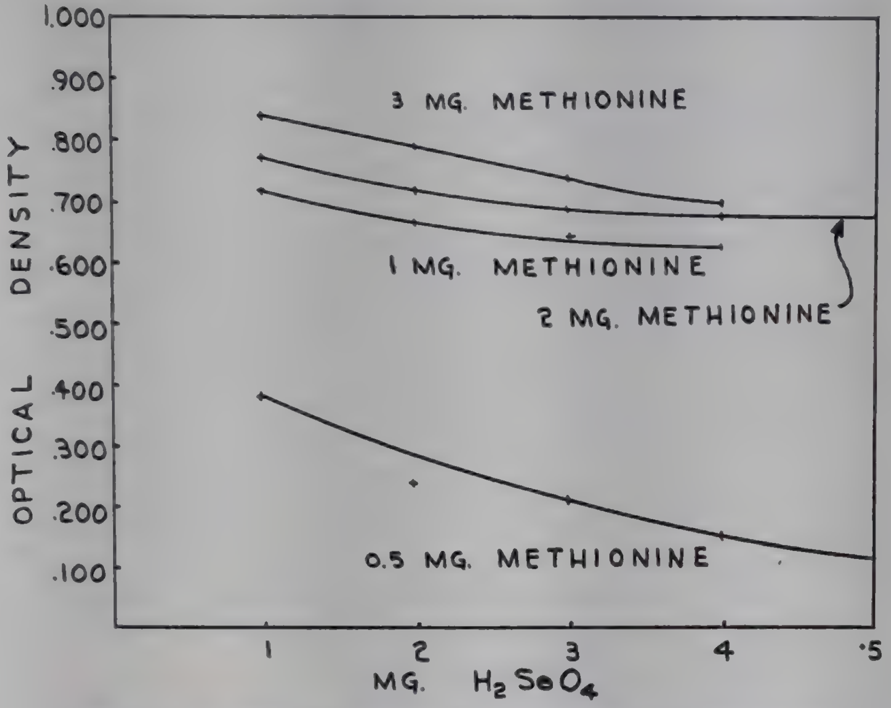


FIG. 1. Reversal of selenate inhibition by DL-methionine

TABLE III

Effect of Methionine-Free Casein Hydrolysate upon Selenate Inhibition in Yeast

Incubation time, 71 hours.

H_2SeO_4	DL-Methionine	Methionine-free hydrolysate	Casein hydrolysate	Optical density
mg.	*mg.*	*mg.*	*mg.*	
0	0	0	0	1.32
1	0	0	0	0.000
1	2	0	0	1.03
0	0	10	0	1.27
1	0	10	0	0.00
1	2	10	0	1.16
0	0	0	28.5	1.48
1	0	0	28.5	0.41
1	2	0	28.5	1.34

In Vitro Oxidation of Methionine by Selenic Acid

The possibility exists that the mode of action of selenate as a toxic reagent for organisms rests on the oxidative capacity of the selenate. Methionine is known to be oxidized readily to the sulfoxide (18) or the sulfone (19) with various oxidizing agents. These have been shown to repress the growth of *Lactobacillus arabinosus* and *Lactobacillus casei* (20, 21). Similarly, the rat has been shown incapable of substituting the sulfone for methionine in the diet (22). If this is true for selenate then its action could be explained by its prevention of methionine utilization. An attempt was therefore made to oxidize methionine with selenic acid. It was found that methionine underwent a ready reaction with selenic acid at room temperature with the rate depending upon the concentrations of starting materials. A crystalline substance containing Se, N, and S was isolated and a description of the procedure will be dealt with in a future paper.

TABLE IV

Specificity of L-*Methionine in Selenate Inhibition*

Incubation time, 67 hours.

H_2SeO_4	DL-Methionine	Optical density	L-Methionine	Optical density	D-Methionine	Optical density
mg.	*mg.*		*mg.*		*mg.*	
0	0	1.120				
1	0	0.000	0.25	0.558	0.25	0.000
1	0.25	0.494	0.50	0.881	0.50	0.000
1	0.50	0.828	1.00	0.910	1.00	0.000
1	1.00	0.839	2.00	0.981	2.00	0.000
1	2.00	0.980				
2	0.25	0.187	0.25	0.257	0.25	0.000
2	0.50	0.770	0.50	0.980	0.50	0.000
2	1.00	0.805	1.00	0.850	1.00	0.000
2	2.00	0.860	2.00	0.920	2.00	0.000
3	0.25	0.000	0.25	0.045	0.25	0.000
3	0.50	0.070	0.50	0.144	0.50	0.000
3	1.00	0.655	1.00	0.720	1.00	0.000
3	2.00	0.710	2.00	0.760	2.00	0.000

Specificity of L-*Methionine in Reversal of Selenate*

The postulate has recently been made (23) that there is present in mammalian tissue a heat-labile factor or factors, presumably an enzyme, which is responsible for the decomposition of selenate and selenite. This is based on the observation that fresh tissue can decompose the selenium salts, whereas autoclaved tissue is incapable of such action. We have obtained somewhat similar results with yeast. This action lends to the cell an active rôle in the detoxification mechanism, in which methionine might possibly cooperate. In order to gain evidence on this point, DL-methionine was resolved into its optical isomers and each was tested for its ability to reverse the selenate effect.

The resolution was carried out according to the method of Windus and Marvel (24). The D-methionine obtained had a specific rotation[8] $[\alpha]_D^{25} = +6.88°$ (0.2000 gm. in 25 ml. of H_2O, 2 dm. tube). The L-methionine obtained had a specific rotation[8] $[\alpha]_D^{25} = -5.0$ (0.2008 gm. in 25 ml. of H_2O, 2 dm. tube). Both isomers gave a positive McCarthy-Sullivan test, but *Lactobacillus arabinosus* utilized only the L form (26) with Henderson and Snell's medium for methionine determination (27).

The results listed in Table IV show clearly that only the natural isomer of methionine is capable of reversing the inhibition by selenate, although the racemic mixture is somewhat more than 50 per cent as active as equal amounts of the L form. This might be due to incomplete resolution (*cf.* foot-note 8); but if not, it suggests that the natural isomer promotes the utilization of the unnatural one, an observation which has also been made for glutamic acid in *Lactobacillus arabinosus* (28).

DISCUSSION

The specificity of methionine in the detoxification of selenate by yeast is apparent first from its direct relationship as depicted in Fig. 1. Within limits, the growth of yeast in selenate is directly proportioned to the amount of methionine present. That methionine is the only substance capable of such action is seen both from the numerous substances tested and the experiments with methionine-free casein hydrolysate. It is interesting to note that unlike animal tissues, yeast is incapable of utilizing homocystine and a methyl donor such as choline, betaine, or creatinine. This would indicate that methionine is synthesized by yeast in a different manner from that accomplished by animals. The fact that yeast is able to synthesize its sulfur-containing amino acids from the sulfate provided lends support to this concept.

The results obtained with the optical isomers of methionine indicate that the cell plays an active rôle in the detoxification mechanisms. This is in line with the recent work of Rosenfeld and Beath (23) who postulated the existence of a selenate-decomposing enzyme in mammalian tissue. Although it is not known whether methionine is involved in the latter system, this compound appears to be the agent by which selenate is detoxified in

[8] D-Methionine $[\alpha]_D^{25} = +7.1°$ obtained by Jackson and Block (25), +8.12° (Windus and Marvel (24)); L-methionine $[\alpha]_D^{25} = -7.3°$ (Jackson and Block (25)), −6.9° (from casein, Windus and Marvel (24)), −7.5° (synthetic product, Windus and Marvel (24)).

yeast, and the specificity of the reversal process is of a type usually associated with enzymatic activity.

The mechanism of the toxic effect of selenium salts remains to be solved. It is believed that the selenate must previously be reduced to selenite before inhibition can take place (29, 23). The effect of the selenite upon vital cellular components has not been explained satisfactorily. The succinic dehydrogenase system has been found to be completely inhibited by selenite (29, 30). Potter and Elvehjem (31) have found that O_2 uptake by yeast in glucose, fructose, or mannose but not in lactose or pyruvate is inhibited by selenite. These authors believe that the selenium acts primarily as an inhibitor of the glycolytic system, possibly by restricting the utilization of glutathione. Since the function of the latter is not known, this explanation is of limited value. DuBois, Rhian, and Moxon (32) have been able to reverse the toxic effect of selenate in rats with glutathione. We have not observed this effect with yeast. Finally, it would seem that the succinic dehydrogenase system, if present in yeast, plays a relatively unimportant rôle, since these organisms utilize succinic acid only poorly.

In view of the ready ability of selenic acid to react with methionine *in vitro*, the transformation of the latter into a compound non-utilizable by the cell offers an attractive explanation for the toxic effect of selenate. However, when dilute concentrations of the reactants are used, the reaction is slow at room temperature. This could not therefore explain the immediate inhibition of O_2 uptake in yeast as observed by Potter and Elvehjem (31).

From the observation that methionine never produces complete reversal of the inhibition, it can be inferred that probably more than one system is affected by the selenate.

From a practical point of view, it is of interest to note that methionine affords protection against selenite in rats (33). It is believed that, in the light of the present study, methionine should be investigated in combatting selenium poisoning in farm animals.

SUMMARY

1. Inhibition of yeast growth by selenate has been found to be reversed by the addition of methionine. No other compound tested displayed this reversal.

2. Within limits, the growth of yeast in the presence of selenate is directly proportional to the methionine present.

3. A methionine-free casein hydrolysate was prepared, and found to have no reversing effect. The addition of DL-methionine rendered it active.

4. Normal casein hydrolysate was capable of partially reversing the selenate inhibition. The addition of methionine enhanced this activity.

5. Yeast could not utilize DL-homocystine plus a methyl donor such as choline, betaine, or creatinine to reverse the inhibition. This suggests that methionine is synthesized in a manner different from that in mammalian tissue.

6. An *in vitro* reaction between selenic acid and methionine was found to take place readily at room temperature. A crystalline reaction product was obtained which contained Se, N, and S.

7. Of the optical isomers of methionine, only the naturally occurring L form was active. This indicates active participation of the cell in the detoxification mechanism.

8. A brief discussion is given of previously proposed explanations for the mode of action of selenium compounds.

BIBLIOGRAPHY

1. Hofmeister, F., *Arch. exp. Path. u. Pharmakol.*, **33**, 198 (1893–94).
2. Schultz, J., and Lewis, H. B., *J. Biol. Chem.*, **133**, 199 (1940).
3. Challenger, F., *Chem. and Ind.*, 657 (1935).
4. Moxon, A. L., Schaeffer, A. E., Lardy, H. A., DuBois, K. P., and Olson, O. E., *J. Biol. Chem.*, **132**, 785 (1940).
5. Westfall, B. B., and Smith, M. I., *J. Pharm. and Exp. Therap.*, **72**, 245 (1941).
6. DuBois, K. P., Moxon, A. L., and Olson, O. E., *J. Nutr.*, **19**, 477 (1940).
7. Williams, R. J., and Saunders, D. H., *Biochem. J.*, **28**, 1887 (1934).
8. Kurd-Karrer, A. M., *Am. J. Bot.*, **24**, 720 (1937).
9. Kurd-Karrer, A. M., *Am. J. Bot.*, **25**, 666 (1938).
10. Gilbertson, L. I., and King, G. B., *J. Am. Chem. Soc.*, **58**, 180 (1936).
11. McCarthy, T. E., and Sullivan, M. X., *J. Biol. Chem.*, **141**, 871 (1941).
12. Mueller, J. H., *Science*, **81**, 50 (1935).
13. Fox, S. W., *Science*, **84**, 163 (1936).
14. Levene, P. A., and Bass, L. W., Nucleic acids, American Chemical Society monograph series, New York (1931).
15. du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **128**, p. cviii (1939).
16. du Vigneaud, V., Chandler, J. P., Moyer, A. M., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939).
17. Baernstein, H. D., *J. Biol. Chem.*, **97**, 663 (1932).
18. Toennies, G., and Kolb, J. J., *J. Biol. Chem.*, **128**, 399 (1939).
19. Toennies, G., and Kolb, J. J., *J. Biol. Chem.*, **140**, 131 (1941).
20. Borek, E., and Waelsch, H., *Arch. Biochem.*, **14**, 143 (1947). Borek, E., Miller H. K., Sheiness, P., and Waelsch, H., *J. Biol. Chem.*, **163**, 347 (1946). Borek, E., Sheiness, P., and Waelsch, H., *Federation Proc.*, **5**, 123 (1946).
21. Waelsch, H., Owades, P., Miller, H. K., and Borek, E., *J. Biol. Chem.*, **166**, 273 (1946).
22. Bennett, M. A., *J. Biol. Chem.*, **141**, 573 (1941).
23. Rosenfeld, I., and Beath, O. A., *J. Biol. Chem.*, **172**, 333 (1948).
24. Windus, W., and Marvel, C. S., *J. Am. Chem. Soc.*, **53**, 3490 (1931).
25. Jackson, R. W., and Block, R. J., *J. Biol. Chem.*, **122**, 425 (1938).
26. Dunn, M. S., Camien, M. N., Shankman, S., and Block, H., *J. Biol. Chem.*, **163**, 577 (1946).

27. Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172**, 15 (1948).
28. Hac, L. R., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **159**, 273 (1945).
29. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **117**, 341 (1937).
30. Stotz, E., and Hastings, A. B., *J. Biol. Chem.*, **118**, 479 (1937).
31. Potter, V. R., and Elvehjem, C. A., *Biochem. J.*, **30**, 189 (1936).
32. DuBois, K. P., Rhian, M., and Moxon, A. L., *Proc. South Dakota Acad. Sc.*, **19**, 71 (1939).
33. Lewis, H. B., Schultz, J., and Gortner, R. A., Jr., *J. Pharm. and Exp. Therap.*, **68**, 292 (1940).

THE IN VITRO SYNTHESIS OF CHOLESTEROL FROM ACETATE BY SURVIVING ADRENAL CORTICAL TISSUE*

By PAUL A. SRERE, I. L. CHAIKOFF, AND W. G. DAUBEN

(*From the Division of Physiology of the Medical School, and the Department of Chemistry, University of California, Berkeley*)

(Received for publication, July 8, 1948)

The prominence of the liver as a site of cholesterol synthesis has been demonstrated by Bloch and his associates (1, 2). Recent observations in this laboratory have shown, however, that the conversion of acetic acid to cholesterol can occur in the hepatectomized rat.[1] In seeking to localize the extrahepatic tissue or tissues in which this synthesis occurs, attention was at first directed to the adrenal cortex because of its high cholesterol content (3). That this tissue is indeed a site of active cholesterol synthesis is borne out by the findings presented here.

EXPERIMENTAL

Preparation and Treatment of Adrenal Cortical Slices—Beef adrenal glands were used in the present investigation. The glands were excised at the abattoir 15 to 20 minutes after the animals had been killed, immediately wrapped in water-proof cellophane, and immersed in ice water for transport to the laboratory. The glands were first freed of extraneous tissue, split in half, and then carefully demedullated. The cortex was next sliced freehand with a razor blade and the slices transferred to a dish containing cold (0°) bicarbonate-Ringer's solution prepared according to Krebs and Henseleit (4). 500 mg. of slices were gently blotted on moist filter paper, weighed quickly, and transferred to a 50 cc. glass-stoppered Erlenmeyer flask containing 5 cc. of the bicarbonate-Ringer's solution and 0.5 cc. of 0.024 N $C^{14}H_3C^{14}OONa$ having 9.4×10^5 counts per minute per cc.[2] The bicarbonate-Ringer's solution was saturated with a gas mixture consisting of 5 per cent CO_2 and 95 per cent O_2, and its pH adjusted to 7.4 to 7.5 just before the addition of the slices. The atmosphere in the flasks was saturated with the same gas mixture immediately before and $1\frac{1}{2}$ hours

---

* Aided by a grant from the American Cancer Society recommended by the Committee on Growth of the National Research Council, and the Cancer Committee of the University of California.

[1] Burstein, L. S., Srere, P. A., and Chaikoff, I. L., unpublished observations.

[2] We are indebted to Dr. H. A. Barker for the doubly labeled acetate used in this investigation. It was prepared from $C^{14}O_2$ with the aid of *Clostridium thermoaceticum* (5).

after the flasks were placed in a constant temperature water bath maintained at 37.5°. The slices were incubated for 3 hours.

Isolation of Cholesterol—At the end of the incubation period the contents of the flasks were transferred to an alcoholic KOH solution (15 gm. of KOH in 50 cc. of 95 per cent ethyl alcohol for each 10 gm. of tissue) and the mixture refluxed for 8 hours on a steam bath. The alcohol was evaporated at steam bath temperature and the alkaline hydrolysate exhaustively extracted with petroleum ether. To insure efficient extraction of the lipides, it was found necessary to keep the ratio of petroleum ether to the alkaline hydrolysate 10:1. Four extractions proved sufficient to remove all of the non-saponifiable fraction; the amount of digitonin-precipitable material extractable from the alkaline hydrolysate was negligible after it had been subjected to four extractions with petroleum ether. The petroleum ether extracts were combined, dried over Na_2SO_4, filtered, and evaporated to dryness on a steam bath. The residue was then dissolved in a minimum amount of hot ethyl alcohol, and an excess of 1 per cent digitonin (in 80 per cent ethyl alcohol) was added. The mixture was heated for an additional 5 minutes and then allowed to stand overnight at 5°. The digitonide was filtered and then washed successively with 85 per cent alcohol, 1:2 acetone-ether mixture, and finally with anhydrous ether. The digitonide was dried and stored in a vacuum desiccator. The C^{14} content of the digitonide was then determined.

The digitonide was cleaved by the pyridine method (6). The crude cholesterol obtained was acetylated with acetic anhydride and the acetate recrystallized once from aqueous methanol. The radioactive cholesteryl acetate obtained was weighed and mixed with a known amount of non-radioactive cholesteryl acetate (the dilution factor was 11.7). The mixture was then recrystallized twice from aqueous methanol. The C^{14} content of the cholesteryl acetate was then determined.

The cholesteryl acetate was saponified with methanolic sodium hydroxide and the cholesterol isolated by dilution with water. The cholesterol isolated was weighed and its C^{14} content determined.

Isolation of Fatty Acids—The aqueous alkaline fraction that remained after the petroleum ether extractions was made acid to bromocresol green and the precipitate allowed to settle. This precipitate has been shown by Sperry *et al.* (7) to contain all the fatty acids of the fraction. In confirmation of Sperry's observation, it was found that the aqueous filtrate contained negligible amounts of ethyl ether-extractable material or of C^{14}. The supernatant was decanted and the residue filtered. The precipitate obtained was exhaustively extracted three times with acetone. The acetone extracts were then combined and evaporated to dryness in the presence of a CO_2

atmosphere, and the residue obtained was extracted with hot petroleum ether. The petroleum ether-insoluble fraction was now redissolved in acetone, and the above procedure repeated. The two petroleum ether extracts were combined, dried over Na_2SO_4, and filtered. The fatty acid content in the petroleum ether extract was determined by weight and titration. The C^{14} content of these fatty acids was determined as described below.

Determination of C^{14}—Samples were oxidized by the method of Van Slyke and Folch (8). The $C^{14}O_2$ evolved was collected as $BaC^{14}O_3$ and its C^{14} content determined by the method of Dauben *et al.* (9).

TABLE I

Recovery of C^{14} *in Fractions Isolated from Beef Adrenal Cortex after Incubation with* $C^{14}H_3C^{14}OOH$

Experiment No.	Adrenal cortex tissue in bath	C^{14} acetate added to Ringer's solution	Fraction or compound isolated	Total counts recovered in compound or fraction	Specific activity	Per cent of added C^{14} recovered in compound or fraction
	gm.	*counts*				
1	21	1.9×10^7	Cholesterol digitonide	3.5×10^4	1840*	0.2
			Fatty acids	2.0×10^5	400	1.0
			Aqueous residue	8.4×10^5		4.4
2	11	9.4×10^6	Cholesterol digitonide	3.4×10^4	1830*	0.4
			Fatty acids	2.8×10^4	150	0.3
			Aqueous residue	5.3×10^5		5.6

* Refers to counts per minute per mg. of its cholesterol.

Results

After incubation, the adrenal cortex tissue and the contents of the flasks were separated into three fractions: cholesterol, fatty acids, and aqueous residue. The C^{14} recovered in these fractions is recorded in Table I. In the first experiment 0.2 per cent of the added C^{14} was converted to cholesterol and 1.0 per cent to fatty acids; in the second experiment 0.4 and 0.3 per cent, respectively, were so converted.

The melting points found for cholesterol and its acetate were in good agreement with reported values (Table II).

The values for the specific activities of cholesterol and of its two derivatives, the acetate and the digitonide, are recorded in Table II. The specific activity of the digitonide is expressed as counts per minute per mg. of the digitonide in Column 4 and as counts per minute per mg. of its cholesterol in Column 6.

Since non-radioactive cholesteryl acetate had been added to the radioactive cholesteryl acetate isolated from the adrenal cortex, it was necessary to correct the values obtained for the specific activity of the acetate and of the cholesterol by the dilution factor 11.7 in order to compare their specific activities with that of the digitonide. The corrected values are recorded in Column 6 of Table II.

TABLE II

Showing Degree of Constancy in Specific Activity of Recrystallized Cholesterol and of its Derivatives

Compound isolated (1)	Melting point		Specific activity		
	Determined (2)	Literature (3)	Expressed as counts per min. per mg. of compound isolated (4)	Expressed as counts per min. per mg. of cholesterol in compound isolated (5)	Expressed as counts per min. per mg. of isolated adrenal cortex cholesterol (6)
	°C.	°C.			
Cholesterol digitonide			4.4×10^2	1.8×10^3	1.8×10^3
Cholesteryl acetate	113–114	115–116	1.4×10^2	1.6×10^2	1.9×10^3
Cholesterol	147–148.5	148–149	1.4×10^2	1.4×10^2	1.7×10^3

DISCUSSION

The constancy of the specific activities of cholesterol and of its two derivatives, the acetate and the digitonide (Column 6, Table II), whose purity had been established (Column 2, Table II), provides conclusive evidence that the C^{14} of the doubly labeled acetic acid had been incorporated into cholesterol of the adrenal cortex.

The demonstration that an isolated surviving tissue can convert acetate to cholesterol was first shown for the liver by Bloch and his associates (1). He also reported that no such synthesis took place in slices of kidney, testis, spleen, and gastrointestinal tract (1). The results of the present investigation demonstrate that another tissue, namely one concerned with the secretion of a steroidal hormone, is capable of converting the common metabolic intermediate acetate to cholesterol.

SUMMARY

The conversion of C^{14}-labeled acetate to cholesterol by surviving slices of beef adrenal cortex is demonstrated. The presence of C^{14} in the cholesterol molecule was established by the finding of a constant specific activity in the cholesterol and in two of its derivatives, the digitonide and the acetate.

BIBLIOGRAPHY

1. Bloch, K., Borek, E., and Rittenberg, D., *J. Biol. Chem.*, **162**, 441 (1946).
2. Bloch, K., *Physiol. Rev.*, **27**, 574 (1947).
3. Long, C. N. H., Recent progress in hormone research, New York, **1**, 99 (1947).
4. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33 (1932).
5. Barker, H. A., and Kamen, M. D., *Proc. Nat. Acad. Sc.*, **31**, 219 (1945).
6. Schoenheimer, R., and Dam, H., *Z. physiol. Chem.*, **215**, 59 (1933).
7. Sperry, W. M., Waelsch, H., and Stoyanoff, V. A., *J. Biol. Chem.*, **135**, 281 (1940).
8. Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, **136**, 509 (1940).
9. Dauben, W. G., Reid, J. C., and Yankwich, P. E., *Anal. Chem.*, **19**, 828 (1947).

THE SPECIFICITY OF LEUCINE AMINOPEPTIDASE*

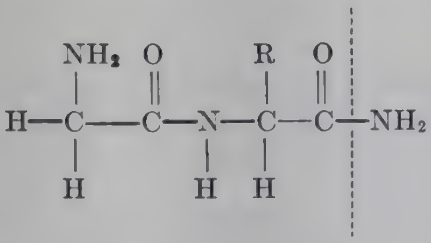
By EMIL L. SMITH and N. BALFOUR SLONIM†

(From the Laboratory for the Study of Hereditary and Metabolic Disorders, and the Departments of Medicine and Biological Chemistry, University of Utah School of Medicine, Salt Lake City)

(Received for publication, June 29, 1948)

Since Linderstrøm-Lang's demonstration that the hydrolysis of L-leucylglycine (LG) is due to a distinct leucyl peptidase (1), various studies have shown that this enzyme is widely distributed (2–4) and requires for its activity the presence of Mn^{++} or Mg^{++} ions (2, 5). The enzyme has been regarded as a typical aminopeptidase (5), since it does not hydrolyze acylated compounds such as benzoyl-L-leucylglycine, and since, in addition to the dipeptide, it hydrolyzes L-leucinamide (LA) and the tripeptides, L-leucylglycylglycine (LGG) and L-leucyl-L-leucylglycine.

It has now been observed that a highly purified preparation of leucine aminopeptidase from hog intestinal mucosa can hydrolyze glycyl-L-leucinamide (GLA). Under the conditions of our experiments, the reaction ceases after the decomposition of a single peptide bond. Since glycyl-L-leucine is not appreciably hydrolyzed, the hydrolysis must occur at the terminal amide bond, as indicated by the dotted line; R represents the isobutyl side chain. Thus, the products of the reaction must be glycyl-L-leucine and ammonia. If the action occurred at the other peptide bond, liberating glycine and LA, the second peptide bond would also be split, since LA is rapidly hydrolyzed by the enzyme. The observation that GLA is hydrolyzed by leucine aminopeptidase has been possible only because the purification process has removed the glycyl-L-leucine dipeptidase. With crude extracts of hog intestinal mucosa and other tissues, the consecutive hydrolysis of both peptide bonds occurs (6).



$$\mathrm{H{-}\underset{H}{\overset{NH_2}{C}}{-}\overset{O}{\overset{\|}{C}}{-}\underset{H}{N}{-}\underset{H}{\overset{R}{C}}{-}\overset{O}{\overset{\|}{C}}\,\vdots\,{-}NH_2}$$

Table I shows that the hydrolysis of GLA follows the kinetics of a first

---

[*] This investigation was aided by a grant from the United States Public Health Service.

[†] Postdoctorate Fellow of the United States Public Health Service.

TABLE I

Specificity of Leucine Aminopeptidase

The enzyme was incubated at 40° in veronal buffer at pH 7.8 to 8.0 with 0.01 M Mn^{++} for 3 hours prior to addition to the buffered substrate solution (0.05 M). The proteolytic coefficient $C = K/E$, where K is the first order velocity constant for the enzyme concentration E expressed in mg. of protein N per cc. of test solution. Two different enzyme preparations of somewhat different activities were used; these are distinguished by the letters in parentheses following the substrates.

Substrate	Enzyme concentration	Time	Hydrolysis	C	C, average
	γ protein N per cc.	*hrs.*	*per cent*		
L-Leucinamide (*a*)	0.8	0.5	19	3.7	3.9
		1.0	36	4.1	
		1.5	46	3.8	
		2.0	58	4.0	
		2.5	66	3.9	
Glycyl-L-leucinamide (*a*)	0.8	0.5	13	2.5	2.2
		1.0	18	1.8	
		1.5	30	2.2	
		2.0	39	2.2	
		2.5	46	2.2	
L-Leucylglycine (*a*)	0.8				5.5
L-Leucylglycylglycine (*a*)	0.8				6.5
Glycyl-L-leucine (*a*)	0.8	3.0	0		
"	4.0	3.0	2		
Diglycylglycine (*a*)	4.0	3.0	0		
L-Leucinamide (*b*)	1.31				2.3
Carbobenzoxy-L-leucinamide (*b*)	6.55	20	1		
Glycyl-L-leucinamide (*b*)	1.31				1.5
Carbobenzoxyglycyl-L-leucinamide (*b*)	6.55	20	1		
L-Glutamyl-L-leucinamide (*b*)	1.31				1.5
Carbobenzoxy-L-glutamyl-L-leucinamide (*b*)	6.55	20	3		
L-Leucyl-L-glutamic acid (*b*)	1.31				1.1
Glycylglycyl-DL-leucylglycine* (*b*)	6.55				0.13
Carbobenzoxyglycylglycyl-L-leucinamide (*b*)	6.55	20	1		
Carbobenzoxy-L-leucylglycylglycine (*b*)	6.55	20	0		
Carbobenzoxy-L-leucylglycine (*b*)	6.55	20	0		

* Used at 0.1 M. We are indebted to Dr. J. S. Fruton for a sample of this compound.

order reaction similar to those of the other substrates.[1] The hydrolysis of all of the sensitive compounds is strongly activated by Mn^{++}, and the activation of the enzyme by Mn^{++} follows the same type of time reaction for GLA as for the other substrates (5).

---

[1] The hydrolysis of LGG follows the kinetics of a zero order reaction. The approximate initial first order constant is given for comparison with the other substrates.

Because of our finding that leucine aminopeptidase hydrolyzes an amide linkage at a distance from the free amino group, as in GLA, the possibility that the enzyme may possess some endopeptidase activity was investigated. Fruton and Bergmann (7) have reported that chymotrypsin shows a dual specificity and can act on substrates characteristic both of endopeptidase and aminopeptidase specificities. We have, therefore, tested the action of our enzyme on a variety of substrates. These results are also presented in Table I.

It is clear that leucine aminopeptidase does not have any detectable endopeptidase action, since no hydrolysis could be observed with carbobenzoxyglycyl-L-leucinamide, carbobenzoxyglycylglycyl-L-leucinamide, carbobenzoxy-L-glutamyl-L-leucinamide, or other N-acylated peptides. On the other hand, compounds with a free amino group such as L-leucyl-L-glutamic acid and L-glutamyl-L-leucinamide (GlLA) are readily hydrolyzed. With GlLA only one peptide bond is hydrolyzed when the purified enzyme is used. However, with a crude extract of intestinal mucosa, both bonds are rapidly hydrolyzed.

It is of interest that the enzyme preparation has a slow but definite action on glycylglycyl-DL-leucylglycine (GGLG). One must assume that this action is due to the leucine aminopeptidase, since no action was detected on diglycylglycine.

DISCUSSION

It is now possible to define the specificity requirements of leucine aminopeptidase more precisely than heretofore. The data in Table I show that the residues on the carboxyl end of the leucine group have some influence on the rate of hydrolysis. The most rapid action is on the tripeptide (LGG) and the dipeptide (LG). The markedly slower action on L-leucyl-L-glutamic acid as compared with LA indicates some inhibitory effect of the second carboxyl group when it is near the sensitive bond. On the other hand, the general configuration of the moiety attached to the carboxyl end of the leucine residue cannot be highly critical, since it has been demonstrated that L-leucyl-β-alanine is rapidly hydrolyzed by this enzyme (8).

The finding of a rapid action on GLA and GlLA was somewhat unexpected. Nevertheless, in the presence of the free amino group, 1 residue removed from the sensitive peptide bond does reduce the rate of hydrolysis by about 40 per cent as compared with LA. The much slower action on the tetrapeptide, GGLG, indicates that the sensitivity of the substrate is greatly decreased as the distance between the sensitive peptide bond and the free amino group is increased.

Leucine aminopeptidase has hitherto been regarded as the prototype of an aminoexopeptidase (9). This concept must now be revised to include our finding that the free amino group need not be present on the leucine residue which possesses the sensitive peptide bond. It has been recently suggested (10) that the rôle of heavy metals in peptidase action is the formation of a coordination compound linking the enzyme and the substrate. If this is so, one must assume that the ease with which the metal (Mn^{++} or Mg^{++}) can form this bridge is a critical function of the distance from the sensitive bond.

EXPERIMENTAL

The enzyme experiments were performed as described in previous papers from this laboratory (4, 8) by means of the carboxyl titration method of Grassmann and Heyde (11). The leucine aminopeptidase was purified in the manner described by Smith and Bergmann (5). Some further purification was achieved by precipitation of the enzyme with 33 per cent acetone at room temperature, followed by dialysis and removal of the inactive precipitate. This procedure gave preparations essentially free of glycyl-L-leucine dipeptidase and tripeptidase activity as measured on diglycylglycine and prolidase.

L-Leucinamide Hydrochloride[2]—A recrystallized preparation of L-leucine methyl ester hydrochloride (10 gm.) was allowed to stand in a pressure bottle at room temperature for 2 days with 50 cc. of anhydrous methanol which had previously been saturated with ammonia gas at 0°. The solution was then repeatedly concentrated *in vacuo* with methanol, and the crystals were filtered and washed with ether. Yield, 8.5 gm. After recrystallization from methanol-ether, thin plates were obtained; m.p. 236–237°.

$C_6H_{15}ON_2Cl$.	Calculated.	C 43.3, H 9.1, N 16.8
166.6	Found.	" 43.3, " 9.1, " 16.9

$[\alpha]_D^{23} = +9.5°$ (5% in water)

Behrens and Bergmann (12) found $[\alpha]_D^{27} = +9.25°$ for the corresponding acetate obtained by hydrogenation of carbobenzoxy-L-leucinamide.

Glycyl-L-leucinamide Hydrochloride

Carbobenzoxyglycyl-L-leucine Methyl Ester—This compound has previously been described as an oil (13). The coupling was performed in the manner given by Stahmann, Fruton, and Bergmann (13) with an ethereal solution of leucine methyl ester prepared in the usual way from 19.6 gm. of the hydrochloride and 26 gm. of carbobenzoxyglycyl chloride. After washing and drying the ethereal solution of the product, it was repeatedly concentrated *in vacuo* with dry ether. Yield, 24 gm. of needles on standing with petroleum ether. After recrystallization from ether-petroleum ether, the melting point was 64–66°.

---

[2] Although this compound has previously been described as the acetate obtained by hydrogenation of carbobenzoxy-L-leucinamide (12), we are prompted to present the much simpler synthesis described above in view of the great utility of LA for studies of the enzymes of various tissues and sera. It should be noted that we have usually obtained somewhat faster enzymatic hydrolysis of the compound obtained by direct amidation than with the acetate prepared through the carbobenzoxy intermediate.

$C_{17}H_{24}O_5N_2$ (336.4). Calculated, N 8.3; found, N 8.3

Carbobenzoxyglycyl-L-leucinamide—The above ester (4 gm.) was amidated in methanol-ammonia in the usual manner. After repeated concentration with methanol, the compound crystallized on standing with a few drops of methanol. Yield, 3.6 gm. After recrystallization from methanol-ether and then from hot water, the melting point was 123–124°.

$C_{16}H_{23}O_4N_3$ (321.3). Calculated, N, 13.1; found, N 13.0, 13.2

Glycyl-L-leucinamide Hydrochloride—The above amide (2.5 gm.) was hydrogenated in the usual manner in the presence of 10 cc. of M HCl, 35 cc. of methanol, and a palladium catalyst. After removal of the catalyst, the solution was concentrated *in vacuo* with ethanol and then with ether. Yield, 1.35 gm. After recrystallization from ethanol-ether, the melting point was 210° (slight browning).

$C_8H_{18}O_2N_3Cl$ (223.7). Calculated, N 18.8; found N 18.8
$[\alpha]_D^{25} = -19.0°$ (5% in water)

L-*Glutamyl*-L-*leucinamide*

Carbobenzoxy-L-glutamyl-L-leucinamide—To a dry ethyl acetate solution of L-leucine methyl ester prepared from 30 gm. of the hydrochloride, there were slowly added 35 gm. of carbobenzoxy-L-glutamic acid anhydride (14). The slightly alkaline solution was allowed to stand at room temperature for 24 hours. It was then washed with dilute hydrochloric acid and with water, dried over Na_2SO_4, and concentrated to a thick oil *in vacuo*. 7 gm. of the oily ester were amidated in methanol-ammonia in the usual manner. After standing at room temperature for 3 days, the solution was concentrated *in vacuo* repeatedly with ether. The residue was extracted into hot ethyl acetate, filtered, and concentrated to dryness. Yield, 3.4 gm.; m.p. 165–169°, after recrystallization from hot water.

$C_{19}H_{27}O_6N_3$ (393.4). Calculated, N 10.68; found, N 10.63

L-*Glutamyl*-L-*leucinamide*—1.0 gm. of the above compound was dissolved in methanol and hydrogenated in the presence of water and acetic acid.

The filtered solution was concentrated repeatedly with absolute ethanol. Yield, 0.5 gm.; m.p. 175–177°.

$C_{11}H_{21}O_4N_3$. Calculated. C 51.0, H 8.2, N 16.2
259.3 Found. " 50.9, " 8.2, " 16.2
$[\alpha]_D^{27} = +6.7°$ (2.1% in water)

L-*Leucyl*-L-*glutamic Acid*—15 gm. of carbobenzoxy-L-leucine hydrazide (15) were converted to the azide and coupled in ethyl acetate with glutamic acid diethyl ester prepared from 14 gm. of the hydrochloride. After standing at room temperature overnight, the ethyl acetate solution was worked up in the usual manner and concentrated *in vacuo*. The oily product was saponified at room temperature in 10 cc. of methanol and 140 cc. of M NaOH for 90 minutes. It was then acidified to Congo red and evaporated to dryness. The carbobenzoxy-L-leucyl-L-glutamic acid was extracted into ethyl acetate, washed with water, extracted into M sodium bicarbonate, and acidified. The product was again dissolved in ethyl acetate, dried over Na_2SO_4, and concentrated *in vacuo*. The substance was hydrogenated in methanol in the usual manner. Water was added during the hydrogenation to dissolve the crystalline peptide. After removal of the catalyst, the solution was concentrated to dryness with ethanol and then with ether. Yield, 1.1 gm.

$C_{11}H_{20}O_5N_2$. Calculated. C 50.7, H 7.7, N 10.8
260.3 Found. " 50.8, " 7.8, " 10.8
$[\alpha]_D^{24} = +10.5°$ (2% in M HCl)

Fischer (16) found the same rotation with this compound prepared from optically active α-bromoisocapronylglutamic acid.

Carbobenzoxyglycylglycyl-L-*leucinamide*—2.5 gm. of carbobenzoxyglycylglycyl-L-leucine methyl ester (17) were amidated in methanol-ammonia in the usual way. After repeated concentration to dryness with methanol, the compound crystallized on gentle warming with water. Yield, 1.7 gm.; m.p. 181–182°.

$C_{18}H_{26}O_5N_4$ (378.4). Calculated, N 14.8; found, N 15.0

SUMMARY

1. Highly purified leucine aminopeptidase of hog intestinal mucosa hydrolyzes glycyl-L-leucinamide, L-glutamyl-L-leucinamide, glycylglycyl-DL-leucylglycine, and L-leucyl-L-glutamic acid in addition to the previously recognized substrates. The hydrolysis of all of these compounds takes place at the carboxyl end of the leucine residue. No endopeptidase action by this enzyme was detected.

2. The concept of aminopeptidase action is revised to include the fact

that the free amino group need not be on the leucine residue which possesses the sensitive peptide bond.

3. The synthesis of a number of new derivatives and peptides of L-leucine is described.

BIBLIOGRAPHY

1. Linderstrøm-Lang, K., *Z. physiol. Chem.*, **182**, 151 (1929); **188**, 48 (1930).
2. Johnson, M. J., and Berger, J., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **2**, 69 (1942).
3. Fruton, J. S., *J. Biol. Chem.*, **166**, 721 (1946).
4. Smith, E. L., *J. Biol. Chem.*, **175**, 553 (1948).
5. Smith, E. L., and Bergmann, M., *J. Biol. Chem.*, **153**, 627 (1944).
6. Smith, E. L., *J. Biol. Chem.*, **176**, 9 (1948).
7. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **145**, 253 (1942).
8. Hanson, H. T., and Smith, E. L., *J. Biol. Chem.*, **175**, 833 (1948).
9. Bergmann, M., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **2**, 49 (1942).
10. Smith, E. L., *J. Biol. Chem.*, **173**, 571 (1948).
11. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183**, 32 (1929).
12. Behrens, O. K., and Bergmann, M., *J. Biol. Chem.*, **129**, 587 (1939).
13. Stahmann, M. A., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **164**, 753 (1946).
14. Bergmann, M., and Zervas, L., *Ber. chem. Ges.*, **65**, 1192 (1932).
15. Bergmann, M., Zervas, L., Fruton, J. S., Schneider, F., and Schleich, H., *J. Biol. Chem.*, **109**, 325 (1935).
16. Fischer, E., *Ber. chem. Ges.*, **40**, 3704 (1907).
17. Fruton, J. S., Smith, V. A., and Driscoll, P. E., *J. Biol. Chem.*, **173**, 457 (1948).

IODINATION OF HYPOPHYSEAL GROWTH HORMONE*

BY CHOH HAO LI, MIRIAM E. SIMPSON, AND HERBERT M. EVANS

(From the Institute of Experimental Biology, University of California, Berkeley)

(Received for publication, July 14, 1948)

It has been shown that if a protein possesses no SH groups, under certain specific conditions it may react with iodine exclusively through the tyrosine residues (1, 2). Since growth hormone as isolated from ox pituitaries contains tyrosine and has no SH groups (3), it would appear interesting to determine whether the tyrosine groups are essential for the growth-promoting action of the hormone after its reaction with iodine. The growth hormone preparations used in the following experiments were isolated by the method previously described (4) and the iodination reactions were carried out at 25°.

Reaction in Acetate Buffer—200 mg. of growth hormone were dissolved in 5 cc. of water with the aid of a few drops of 0.1 N HCl; to the clear solution were added 15 cc. of pH 5.25 acetate buffer of ionic strength 0.10. The suspension was then stirred vigorously while 1 cc. of 0.10 N iodine solution was added drop by drop. The solution was continuously stirred gently. At the end of 20 hours, the solution still had a yellowish tint, indicating the presence of some uncombined iodine. After the excess iodine was removed by a few drops of 0.01 N thiosulfate solution, the solution was first dialyzed against running tap water for 24 hours and then against distilled water for 3 days in a cold room. The iodinated hormone was recovered by lyophilization and the dry material is designated as Preparation A.

In another experiment, conditions were exactly the same as those described above, except that 3.0 cc. of 0.10 N iodine solution were used instead of 1.0 cc. After the removal of the excess iodine with thiosulfate at the end of 20 hours, the solution was thoroughly dialyzed and finally lyophilized. The final product is called Preparation B.

The method of Taurog and Chaikoff (5) was used for iodine determination[1] and the free tyrosine content was determined by the procedure of Lugg (6). Table I presents the analytical results. The theoretical values of iodine were computed from the free tyrosine content by assuming that the lowering of free tyrosine value is due to the formation of diiodotyrosine

---

* Aided by grants from the American Cancer Society through the Committee on Growth of the National Research Council, the United States Public Health Service contract No. RG-409, and the Research Board of the University of California, Berkeley.

[1] We are indebted to J. Wolff for the iodine determination.

residues in the protein molecule. It is evident from Table I that this assumption is probably correct, for the iodine content obtained analytically agrees very well with the theoretical data. This would appear to indicate that no groups other than tyrosine react with iodine. There are eleven tyrosine groups in the molecule of untreated hormone (3). From the analytical data in Table I, it can be shown that nine of the tyrosine residues in Preparation A have not been modified by iodine, whereas four out of eleven tyrosine groups in Preparation B become diiodotyrosine and are no longer free.

Reaction in Urea Solution—If the tyrosine residues in the growth hormone molecule are all available for iodination, it would be expected that Preparation B contains 6.30 mg. per cent of iodine. The results in Table I show that the hormone takes up only 2.30 mg. per cent of iodine when it reacts with an excess amount[2] of iodine at 25° for 20 hours. Under such conditions, tyrosine in the free state is completely iodinated as predicted by kinetic data (7). Hence, it may be assumed that two-thirds of the tyrosine groups in the hormone are not available for iodination. Similar conclusions have been drawn from experiments with pepsin and albumin when it was further found that the unreactive tyrosine residues could be iodinated after denaturation (2).

TABLE I

Tyrosine and Iodine Content of Iodinated Growth Hormone Preparations

Preparation	Tyrosine content	No. of tyrosine groups per mole hormone	Iodine content	
			Found	Theoretical
	per cent		*per cent*	*per cent*
Untreated	4.50	11	0.00	0.00
A	3.66	9	1.18	1.10
B	2.85	7	2.31	2.30
C	1.10	3	4.77	5.30

100 mg. of the hormone were dissolved in 10 cc. of pH 5.25 acetate buffer containing 7.1 M urea and 1.5 cc. of 0.10 N iodine were added. The solution was allowed to react with constant stirring at 25° for 20 hours. It was then dialyzed and lyophilized. The final product (Preparation C) was analyzed for iodine and tyrosine as described above. As shown in Table I, Preparation C contains 5.03 mg. per cent of iodine and 1.10 per cent tyrosine, indicating that even in urea the tyrosine groups are still not com-

---

[2] If we assume that the tyrosine residues (4.5 per cent) in the hormone are completely iodinated to diiodotyrosine, about 1 cc. of 0.10 N iodine per 100 mg. of hormone would be required.

pletely liberated to react with iodine, although a higher uptake of iodine is evident as compared with iodination in solutions without urea. As already noted, the hormone is partially precipitated in pH 5.25 acetate buffer. It was found that the solution becomes clear in the presence of urea.

Bioassay of Iodinated Growth Hormone—Two assay methods were used to estimate the growth-promoting activity of the iodinated preparations: one depends on the body weight increment of hypophysectomized rats and the other on the stimulation of the proximal epiphyseal cartilage of the tibia (8). The results are summarized in Table II. It is evident that the growth-promoting potency of the hormone decreases as the uptake of iodine increases. For instance, a 0.10 mg. daily dose of Preparation B caused only 9.4 gm. of body weight increase in hypophysectomized rats in 10 days, while the untreated hormone gave a value of 18.0 gm. at the same daily dose. When eight out of eleven tyrosine groups in the hormone are iodinated (Preparation C), the biological activity is greatly diminished. It should be mentioned that urea alone does not impair the biological activity of the hormone (4).

TABLE II

Bioassay of Iodinated Growth Hormone Preparations

Preparation	Body growth in 10 days*		Proximal epiphyseal cartilage of tibia in 4 days		
	No. of rats	Average growth	Daily dose	No. of rats	Average width
		gm.	*mg.*		*μ*
Untreated	13	18.0	0.03	8	308
			0.01	8	244
			0.00	8	158
A	8	13.0	0.05	14	304
B	7	9.4	0.05	7	289
C	9	7.4	0.05	6	238

* Daily dose in each case, 0.10 mg.

SUMMARY

The reaction of iodine with the hypophyseal growth hormone has been studied in acetate buffer and urea solution. It was found that the uptake of iodine could be accounted for by decrease in the free tyrosine groups on the assumption that the tyrosine residues in the protein molecule are converted into diiodotyrosine. It would appear that the tyrosine residues, however, are not all available for iodination in acetate buffer and that in the presence of urea more tyrosine groups become available for the reaction. When the iodinated preparations were assayed for growth potency, it was found that the activity decreased as the uptake of iodine increased.

Thus, it may be concluded that tyrosine in the hormone molecule is essential for its growth-promoting activity.

BIBLIOGRAPHY

1. Herriott, R. M., in Anson, M. L., and Edsall, J. T., Advances in protein chemistry, New York, **3,** 169 (1947). Olcott, H. S., and Fraenkel-Conrat, H., *Chem. Rev.*, **41,** 151 (1947).
2. Li, C. H., *J. Am. Chem. Soc.*, **67,** 1065 (1945).
3. Li, C. H., in Annual review of biochemistry, Stanford University, **16,** 291 (1947).
4. Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, **159,** 353 (1945).
5. Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, **163,** 313 (1946).
6. Lugg, J. W. H., *Biochem. J.*, **32,** 775 (1938).
7. Li, C. H., *J. Am. Chem. Soc.*, **64,** 1147 (1942).
8. Li, C. H., and Evans, H. M., in Harris, R. S., and Thimann, K. V., Vitamins and hormones, New York, **5,** 197 (1947).

THE ISOLATION OF PREGNANOL-3(α)-ONE-20, PREGNANEDIOL-3(α),20(β), AND ETIOCHOLANEDIOL-3(α),17(β) FROM THE BILE OF PREGNANT COWS*

By W. H. PEARLMAN and EMILY CERCEO

(*From the Department of Biochemistry, Jefferson Medical College, Philadelphia*)

(Received for publication, July 22, 1948)

After isolating estrone from the unhydrolyzed bile of pregnant cows (2)' the authors decided to investigate the accompanying neutral fraction, for it appeared likely that progesterone metabolites might be contained therein. The determination of the nature and quantity of such compounds in bile would contribute to our knowledge of progesterone metabolism particularly during pregnancy, and might furnish information as to the degree to which the liver participates in steroid hormone metabolism.

For this purpose, two batches of pooled bile specimens were utilized, Batch A (31.0 liters), from which 20 mg. of crude estrone had been isolated (2), and Batch B (33.0 liters). The extraction and initial fractionation procedures were those previously employed (2). The ether-soluble, neutral fraction of unhydrolyzed bile yielded the following crystalline products but the corresponding fraction obtained from the residual bile after acid hydrolysis did not.

The non-digitonin-precipitable, alcoholic ketonic fraction (Batch A) furnished after chromatography 15 mg. of a crystalline product, m.p. 147–148°. It was purified and its identity as pregnanol-3(α)-one-20 established by determination of the melting point before and after admixture with pregnanol-3(α)-one-20, carbon and hydrogen analysis, determination of the specific optical rotation, and the preparation of an acetyl derivative which gave the expected carbon and hydrogen values on analysis and which did not depress the melting point after admixture with pregnanol-3(α)-one-20,3-acetate.

A search was made for progesterone in the non-alcoholic ketonic fraction (Batch A). Examination of this material after chromatography revealed several fractions with intense absorption at 240 mμ. These fractions were combined and induced to crystallize, thereby furnishing Compound X, m.p. 215–218°. It exhibited an ultraviolet absorption spectrum almost identical

---

* This work was supported by grants-in-aid from the United States Public Health Service, under the National Cancer Institute Act, and from Ciba Pharmaceutical Products, Inc., Summit, New Jersey. A preliminary report (1) was presented before the meeting of the American Society of Biological Chemists at Atlantic City, March, 1948.

with that of progesterone (see Fig. 1) but comparison of the melting points and distribution coefficients (see Table II) clearly established its non-identity with progesterone as well as with certain other non-alcoholic, α,β-unsaturated ketones. Compound X was isolated in an amount insufficient for carbon and hydrogen analysis, but as much as 13 mg. may have been contained originally in the non-alcoholic ketonic fraction. This figure is based on the relative extinction coefficients of Compound X and of the fraction from which it was derived.

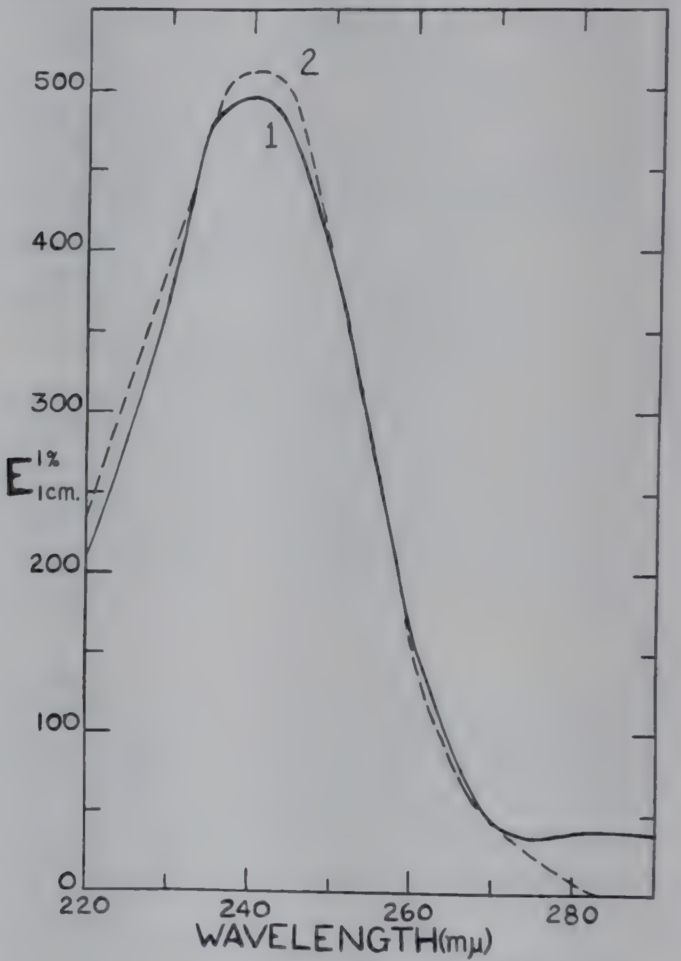


Fig. 1. Ultraviolet absorption spectra of Compound X (Curve 1) and of progesterone (Curve 2). Solvent, absolute ethanol.

The non-ketonic fraction (Batch A) yielded 22 mg. of crude Compound Y and 26 mg. of crude Compound Z. These compounds, in amounts of 26 and 55 mg. respectively, were also isolated from the non-digitonin-precipitable, non-ketonic fraction of Batch B. The products were purified and their identities established as follows. Compound Y, m.p. 235–236°, gave no melting point depression on admixture with pregnanediol-3(α),20(β) prepared from either pregnanol-3(α)-one-20 or progesterone. It was further identified by carbon and hydrogen analysis, specific optical rotation, and the preparation of a diacetyl derivative which did not depress the melting point on admixture with pregnanediol-3(α),20(β),3,20-diacetate. Compound Z, m.p. 228.5–229°, did not depress the melting point on admixture with etiocholanediol-3(α),17(β), exhibited practically the same specific optical rotation, and gave the expected carbon and hydrogen values on analysis. Furthermore, Compound Z yielded on chromic acid oxidation a product which gave no melting point depression on admixture with etiocholanedione-3,17, exhibited the correct specific optical rotation, and gave the expected carbon and hydrogen values on analysis.

EXPERIMENTAL[1]

Collection and Extraction of Bile—Gallbladder bile was freshly collected from slaughtered cows estimated to be at least 5 months pregnant.[2] The bile was refrigerated and extracted within 24 to 48 hours subsequent to collection. Two batches of bile were utilized to obtain the neutral fraction of *unhydrolyzed* bile: Batch A (31.0 liters) was the same collection of bile from which estrone had been isolated in this laboratory (2); Batch B (33.0 liters) was worked up[3] in order to procure additional non-ketonic, neutral products.

Procedures have already been described (2) for obtaining the neutral material; it is ether-soluble and constitutes the 90 per cent methanol phase after repeated partitioning with petroleum ether.

Fractionation of Neutral Material of Unhydrolyzed Bile—Separation into ketonic and non-ketonic moieties was achieved with the aid of Girard's Reagent T(3). The ketones on treatment with succinic anhydride in pyridine (4) were resolved into alcoholic and non-alcoholic fractions. Digitonides were precipitated by treating the alcoholic ketones and the non-ketones respectively with hot 70 per cent methanol containing 1 per cent digitonin; the digitonides were split in the usual way with pyridine and dry ether (5).

Isolation of Pregnanol-3(α)-one-20—The non-digitonin-precipitable, alcoholic ketonic fraction (218 mg. from Batch A) was dissolved in 1.5 ml. of benzene and 1.4 ml. of petroleum ether (b.p. 35–45°) added. The solution was passed through a narrow column containing 4 gm. of alumina[4] previously wetted with the same solvent mixture. The adsorbed material was successively eluted with benzene-petroleum ether, benzene, benzene-ether, ether, ether-methanol, and methanol. The benzene (25 to 75 per cent)-petroleum ether eluates (a total volume of 725 ml. of eluant was employed) contained 25 mg. of nicely crystalline material. On recrystallization from aqueous methanol it gave 15 mg. of needles, m.p. 147–148°. Further recrystallization from the same solvent mixture yielded a product melting sharply at 148–148.5°, $[\alpha]_D^{26} = +108.5° \pm 1°$ (9.23 mg. in 1.23 ml. of absolute ethanol solution). The material was recovered and recrystallized for analysis.

---

[1] All melting points reported are corrected except where otherwise indicated.

[2] We are indebted to Dr. C. E. Mootz of the United States Department of Agriculture, Philadelphia, for arranging a veterinarian-supervised collection of bile. Dr. Irwin Rothman was of great assistance in judging the approximate stage of pregnancy by examination of the fetuses.

[3] Batch B was worked up in a slightly different way. The bile prior to extraction with butanol was made acid to Congo red with concentrated HCl. This resulted in a more rapid separation of the two phases. The butanol extracts were washed with a little water, neutralized with a few drops of concentrated NH_4OH, and evaporated *in vacuo*, etc.

$C_{21}H_{34}O_2$. Calculated, C 79.19, H 10.76; found, C 79.35, H 10.64

There was no depression of the melting point on admixture with an authentic specimen[5] of pregnanol-3(α)-one-20, m.p. 148.5–149°.

Following treatment of 8.6 mg. of the above material, m.p. 147–148°, with acetic anhydride and pyridine for 24 hours at room temperature, a product was obtained which on repeated recrystallization from aqueous methanol gave 4 mg. of plates, m.p. 97–98°.

$C_{23}H_{36}O_3$. Calculated, C 76.62, H 10.06; found, C 76.75, H 10.20

The melting point was not depressed on admixture of this product with pregnanol-3(α)-one-20,3-acetate, m.p. 99–100°, prepared under identical conditions.

The digitonin-precipitable alcoholic ketonic fraction derived from Batch A weighed 44 mg. It was chromatographed, thereby yielding 1 mg. of a slightly impure crystalline product, m.p. 184–186°, which was not identified.

Isolation of Compound X—The non-alcoholic ketonic fraction (152 mg. from Batch A) was dissolved in 3 ml. of benzene and 1.8 ml. of petroleum ether added. The material was adsorbed on a narrow column containing 4 gm. of alumina and eluted successively with benzene-petroleum ether, benzene, benzene-ether, ether, ether-methanol, and methanol. The material (41 mg.) eluted by 125 ml. of ether (25 to 75 per cent)-benzene exhibited a maximum absorption at 240 mμ; $E_{1\,\text{cm.}}^{1\%} = 155$ (in absolute ethanol). Crystallization from ether yielded 5 mg. of a crude product, m.p. 206–212°; repeated recrystallization from the same solvent gave 1.0 mg., m.p. 215–218°. The latter material is referred to as Compound X. Its ultraviolet absorption spectrum is almost identical with that of progesterone (see Fig. 1); $E^{1\%}_{1\text{ cm.}} = 494$ and 509 at 240 mμ for the two compounds respectively. However, Compound X is not identical with progesterone nor with certain other non-alcoholic, α,β-unsaturated ketones because of marked differences in the melting points and distribution coefficients of the respective compounds (see Table I).

---

[4] The alumina employed throughout this study is described as Harshaw, plain activated. Prior to use, it was suspended in glacial acetic acid, filtered, washed with copious amounts of distilled water, dried at 150° for several hours, and finally placed in a tightly sealed container.

[5] Kindly furnished by Dr. Seymour Lieberman. Butenandt and Müller (6) reported pregnanol-3(α)-one-20, m.p. 148–149° (uncorrected), $[\alpha]_D^{19} = +113.9° \pm 1.2°$ (in absolute ethanol); acetyl derivative, m.p. 99° (uncorrected).

Chromatography of the mother liquors (40 mg.) of Compound X did not result in any substantial degree of purification. In fact, an appreciable amount of material absorbing at 240 mμ failed to be recovered in the process. Counter-current distribution of the remaining material seemed to be a more effective means of purification; an additional 1 mg., m.p. 213–217°, was thereby obtained.

TABLE I

Distribution Coefficients: Compound X and Other Non-Alcoholic α,β-Unsaturated Ketones

Substance	M.p.	Distribution coefficient,* petroleum ether-70 per cent methanol
	°C.	
Compound X	215–218	0.05
Progesterone	127–128	0.33
Δ-4-Cholestenone-3	81– 82	3.07
Δ-4-Androstenedione-3,17	173–174	0.07

* Determined by distributing approximately 1 mg. of the substance at room temperature between 50 ml. portions of petroleum ether (previously washed with concentrated H_2SO_4, then water, and distilled at 35–45°) and of 70 per cent methanol; the solvents were mutually saturated prior to use. The residues obtained from each phase were taken up in absolute ethanol and the densities determined at 240 mμ. The ratio of the densities gave the distribution coefficient.

Isolation of Pregnanediol-3(α),20(β) (Compound Y) and of Etiocholanediol-3(α),17(β) (Compound Z)—The non-ketonic fraction (801 mg. from Batch A) was treated with ethyl acetate at room temperature, thereby furnishing 183 mg. of crystalline material, m.p. 203–217°. Recrystallization from the same solvent yielded 142 mg. of a product, m.p. 200–208°, which although nicely crystalline, proved to be a complex mixture. Details of the chromatographic analysis are not furnished in this instance but are described below for the non-ketonic fraction obtained from Batch B. Chromatography was more effectively carried out in the latter instance.

The non-ketonic fraction (Batch A) furnished 22 mg. of impure Compound Y, m.p. 231–235°, which was purified by chromatography. There were finally obtained 5 mg., m.p. 234–236°, $[\alpha]_D^{31} = +18° \pm 4°$ (4.77 mg. in 1.23 ml. of dioxan solution); it crystallized in needles from ethyl acetate and in rectangular plates from ethanol.

The non-ketonic fraction (Batch A) also furnished 26 mg. of somewhat impure Compound Z, m.p. 227–229°, $[\alpha]_D^{29} = -9°$ (in dioxan). Repeated recrystallization from ethyl acetate gave 10 mg. of needles, m.p., 229–229.5°.

$C_{19}H_{32}O_2$. Calculated, C 78.03, H 11.03; found, C 78.15, H 11.07

The non-ketonic fraction (Batch B) after the removal of digitonin-precipitable material (13 mg. of oil) weighed 856 mg. When treated with ethyl acetate, it formed a gel. The material was consequently recovered and taken up in 0.5 ml. of acetone and 3 ml. of benzene were added. The solution was passed through a column (90 × 20 mm.) containing 15 gm. of alumina previously wetted with benzene. A condensed version of the chromatographic analysis is given in Table II.

TABLE II

Chromatographic Analysis of Non-Ketonic Fraction (Non-Digitonin-Precipitable, Batch B)

Fraction No.	Eluant		Eluate	
	Volume	Composition	Weight	Description or compounds isolated
	ml.		*mg.*	
1– 5	115	Benzene	327	Semicrystalline; Compounds Y and Z
6–12	310	"	111	Compound Z
13–15	100	Benzene-ether (25%)	33	" "
16–22	200	" (50–100%)	61	Colorless glass
23–24	50	Ether-methanol (1%)	38	" "
25–26	50	" (5%)	131	Unidentified crystals, 4 mg.; m.p. 253–254°
27–34	215	" (5–50%)	79	Oil
35–37	50	Methanol	5	"

Fractions 1 to 5 (total 327 mg., see Table II) were combined, which contained 46 mg. of benzene-insoluble material which, on treatment with ethanol, yielded 27 mg. of slightly impure Compound Y, m.p. 233–236°. Repeated recrystallization from the same solvent gave 16 mg. of rectangular plates, m.p. 235–236°, $[\alpha]_D^{25} = +19° \pm 5°$ (10.8 mg. in 2.00 ml. of absolute ethanol solution).

$C_{21}H_{36}O_2$. Calculated, C 78.69, H 11.33; found, C 78.73, H 11.25

It gave no melting point depression on admixture with pregnanediol-3(α),20(β), m.p. 235° (rectangular plates from ethanol), which had been prepared in this laboratory by hydrogenation of pregnanol-3(α)-one-20 in glacial acetic acid containing platinum oxide. Comparison of Compound Y was likewise made with a specimen of pregnanediol-3(α),20(β), m.p. 235°, $[\alpha]_D^{27} = +18° \pm 4°$ (6.9 mg. in 1.23 ml. of absolute ethanol solution), which had been prepared in this laboratory from progesterone under the same conditions of hydrogenation as above. A specimen[6] of slightly impure pregnanediol-3(α),20(β), m.p. 234–234.5°, which was kindly furnished by Dr. Miescher, did not depress the melting point on admixture with our synthetic preparation. On the other hand, admixture of Compound Y, m.p. 235–236°, with pregnanediol-3(α),20(α), m.p. 238–239°, gave a melting point depression of 22–26°.

Treatment of 11 mg. of Compound Y in pyridine and acetic anhydride at room temperature for 24 hours gave 13 mg. of a product which could not be crystallized from methanol. It crystallized, however, from pentane on careful chilling in an alcohol-solid CO_2 bath; further recrystallization from the same solvent gave 5 mg. of needles, m.p. 109–109.5°. It did not depress the melting point on admixture with an authentic specimen[6] of pregnanediol-3(α),20(β),3,20-diacetate, m.p. 110–110.5°.

A small amount (3 mg.) of Compound Z, m.p. 225–227°, was obtained on chromatographic analysis of the benzene-soluble material (281 mg.) contained in Fractions 1 to 5 (Table II). Fractions 6 to 15 (Table II) yielded more of this compound on treatment with ethanol, 52 mg. of needles, m.p. 223–227°, being obtained. Repeated recrystallization from the same solvent gave 30 mg. of pure Compound Z, m.p. 228.5–229°, $[\alpha]_D^{25} = -1.6° \pm 1.4°$ (30.1 mg. in 3.00 ml. of absolute ethanol solution).

$C_{19}H_{32}O_2$. Calculated, C 78.03, H 11.03; found, C 78.12, H 11.05

It did not depress the melting point on admixture with a specimen[7] of etiocholanediol-3(α),17(β), m.p. 226–227°, $[\alpha]_D^{28} = 0 \pm 1.8°$ (in ethanol). Acetyl and benzoyl derivatives of Compound Z were prepared but these failed to crystallize.

To a solution of 12.0 mg. of Compound Z, m.p. 225.5–227°, in 1 ml. of glacial acetic acid was added 0.8 ml. of 1 per cent CrO_3 in the same solvent.

---

[6] Dr. K. Miescher gave as the melting points of the specimens of pregnanediol-3(α),20(β) and its diacetyl derivative 240–241° and 113–115° respectively. These observations (*cf.* (7)) were apparently made under the microscope according to Kofler, whereas in this laboratory melting point determinations are performed on the specimen in a capillary tube immersed in an oil bath containing a long stem standardized thermometer.

[7] This specimen was kindly furnished by Dr. H. L. Mason who with Dr. J. J. Schneider obtained it on incubating etiocholanol-3(α)-one-17 with liver slices (8). Dr. Mason in a private communication stated that he likewise was unsuccessful in preparing a crystalline acetate or benzoate.

The reaction mixture was allowed to stand at room temperature for 24 hours and, after adding a little methanol, was poured into water and extracted with ether. The ether solution was washed successively with dilute HCl, $NaHCO_3$ solution, and finally with water. Evaporation of the ether yielded 13 mg. of material which gave, on repeated recrystallization from ether-pentane, 4 mg. of plates, m.p. 132–133°, $[\alpha]_D^{24} = +123° \pm 8°$ (3.13 mg. in 1.23 ml. of absolute ethanol solution). The material was recovered for analysis.

$C_{19}H_{28}O_2$. Calculated, C 79.13, H 9.78; found, C 79.44, H 9.90

It gave no melting point depression on admixture with a specimen[8] of etiocholanedione-3,17, m.p. 132°. It gave a melting point depression of 20–22° on admixture with a specimen[9] of androstanedione-3,17, m.p. 131.5–132°.

DISCUSSION

The isolation from bile of pregnanol-3(α)-one-20, a known (10) metabolite of progesterone, implicates the liver in the metabolism of progesterone. This view is supported by a substantial amount of indirect evidence (*e.g.*, (11, 12)) accumulated in recent years which indicates that the liver is an important if not major site of progesterone inactivation. Pregnanol-3(α)-one-20 is a characteristic constituent of the urine of pregnancy (13–15) but has hitherto not been sought for in bile.

The accompanying product, pregnanediol-3(α),20(β), which is excreted in bile but curiously enough not in urine, may be considered to be a metabolite of pregnanol-3(α)-one-20 inasmuch as it can readily be prepared by catalytic hydrogenation of the latter substance. Its stereoisomer, pregnanediol-3(α),20(α), which is definitely known[10] to be a metabolite of progesterone and is a major urinary steroid of the pregnant cow (and of many but not all species), is conspicuous by its absence from the bile of this species. However, in a study (17) in which massive doses of pregnenol-3(β)-one-20 were administered orally to a postmenopausal woman, minute amounts of pregnanediol-3(α),20(α) were obtained from the bile. Whether pregnanediol-3(α),20(α) is a normal constituent of the bile of pregnant women remains to be investigated. Another stereoisomer of pregnanediol occurring in bile but not in urine is allopregnanediol-3(β),20(β); this substance was previously obtained from ox bile (18) but failed to be isolated in this instance from cow bile.

[8] This specimen was kindly furnished by Dr. Erwin Schwenk. Ercoli and Mamoli (9) reported etiocholanedione-3,17, m.p. 131–132°, $[\alpha]_D^{18} = +113°$ (ethanol).

[9] Kindly furnished by Dr. Seymour Lieberman.

Although products related to progesterone metabolism were obtained from bile, progesterone itself was not isolated. However, another substance (Compound X) which, like progesterone, appears to be an α,β-unsaturated, non-alcoholic ketone, was isolated. Unfortunately, not enough material was available for further structural elucidation. In this connection, it might be well to point out that progesterone can barely be detected in pregnancy urine (19) although its reduction products are abundantly present therein; the progesterone level in blood (20) is likewise estimated to be very low.

Etiocholanediol-3(α), 17(β) has not been isolated from urine although its 17-epimer, etiocholanediol-3(α), 17(α), is known (20–23) to be present. Both compounds are formed following the incubation of etiocholanol-3(α)-one-17 with surviving rabbit liver slices (8). The same reactions probably occur *in vivo* in view of the isolation of etiocholanediol-3(α), 17(β) from bile; the *in vivo* formation of etiocholanediol-3(α), 17(α) from dehydroisoandrosterone has recently (23) been demonstrated.

The presence in pregnancy bile of a C_{19} steroid (which incidentally is probably devoid of androgenic activity) is not altogether surprising since other compounds in this category, such as dehydroisoandrosterone and androsterone, are found (24, 25) in pregnancy urine. It is remarkable, however, that the ratio of the quantities of C_{19} steroid to C_{21} steroid (progesterone reduction products) is so high in the former case. Pertinent to this observation is the recent finding (26) that the pregnant cow excretes considerable quantities of androgenic material in the feces. It is of interest also that the neutral compounds of cow bile, including the estrogens (2), are excreted as such, whereas in the urine of most species these or related products exist predominantly in a conjugated form.

SUMMARY

Pregnanol-3(α)-one-20, pregnanediol-3(α), 20(β), and etiocholanediol-3(α), 17(β) were obtained in amounts ranging from approximately 0.5 to 2 mg. per liter from gallbladder bile of cows in an advanced stage of pregnancy. An unidentified substance (Compound X) was also isolated but in an amount insufficient for analysis; it is not identical with progesterone although it possesses some of its chemical features. The foregoing products were found in the neutral fraction of unhydrolyzed bile; the corresponding fraction of the residual bile after acid hydrolysis failed to yield any crystalline material.

A discussion has been presented which deals with the significance of these

---

[10] For a discussion of the intermediary metabolism of progesterone (and other steroid sex hormones) see Pincus and Pearlman (16).

findings, particularly as it pertains to the rôle of the liver in steroid hormone metabolism.

The authors are indebted to Mr. James Rigas for the microanalyses.

BIBLIOGRAPHY

1. Pearlman, W. H., *Federation Proc.*, **7**, 178 (1948).
2. Pearlman, W. H., Rakoff, A. E., Cantarow, A., and Paschkis, K. E., *J. Biol. Chem.*, **170**, 173 (1947).
3. Girard, A., and Sandulesco, G., *Helv. chim. acta*, **19**, 1095 (1936).
4. Dutcher, J. D., and Wintersteiner, O., *J. Am. Chem. Soc.*, **61**, 1992 (1939).
5. Schoenheimer, R., and Dam, H., *Z. physiol. Chem.*, **215**, 59 (1933).
6. Butenandt, A., and Müller, G., *Ber. chem. Ges.*, **71**, 191 (1938).
7. Meystre, C., and Miescher, K., *Helv. chim. acta*, **29**, 33 (1946).
8. Schneider, J. J., and Mason, H. L., *Federation Proc.*, **7**, 184 (1948); *J. Biol. Chem.*, **175**, 231 (1948).
9. Ercoli, A., and Mamoli, L., *Ber. chem. Ges.*, **71**, 156 (1938).
10. Dorfman, R. I., Ross, E., and Shipley, R. A., *Endocrinology*, **42**, 77 (1948).
11. Kochakian, C. D., Haskins, I. L., and Bruce, R. A., *Am. J. Physiol.*, **142**, 326 (1944).
12. Masson, G., and Hoffman, M. M., *Endocrinology*, **37**, 111 (1945).
13. Marker, R. E., and Kamm, O., *J. Am. Chem. Soc.*, **59**, 1373 (1937).
14. Marker, R. E., and Rohrmann, E., *J. Am. Chem. Soc.*, **61**, 3476 (1939).
15. Pearlman, W. H., and Pincus, G., *Federation Proc.*, **1**, 66 (1942).
16. Pincus, G., and Pearlman, W. H., in Harris, R. S., and Thimann, K. V., Vitamins and hormones, New York, **1**, 293 (1943).
17. Pearlman, W. H., and Pincus, G., *Federation Proc.*, **5**, 79 (1946).
18. Pearlman, W. H., *J. Biol. Chem.*, **166**, 473 (1946); *J. Am. Chem. Soc.*, **66**, 806 (1944).
19. Loewe, S., and Voss, H. E., *Arch. Gynäk.*, **158**, 364 (1934).
20. Haskins, A. L., *J. Clin. Endocrinol.*, **1**, 65 (1941).
21. Butenandt, A., Tscherning, K., and Dannenberg, H., *Z. physiol. Chem.*, **248**, 205 (1937).
22. Dorfman, R. I., Cook, J. W., and Hamilton, J. B., *J. Biol. Chem.*, **130**, 285 (1939).
23. Miller, A. M., and Dorfman, R. I., *Endocrinology*, **42**, 174 (1948).
24. Marker, R. E., and Lawson, E. J., *J. Am. Chem. Soc.*, **60**, 2928 (1938).
25. Marker, R. E., *J. Am. Chem. Soc.*, **61**, 944 (1939).
26. Longwell, B. B., and Gassner, F. X., *Federation Proc.*, **6**, 272 (1947).

THE KINETICS OF THE OXIDATION OF BENZOIC ACID BY CERTAIN MYCOBACTERIA

BY G. S. EADIE, FREDERICK BERNHEIM, AND ROBERT J. FRITZGERALD

(From the Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina)

(Received for publication, July 22, 1948)

Benzoic acid is oxidized by certain *Mycobacteria* (1). They do not oxidize it completely to carbon dioxide and water; the oxygen uptake usually stops when 5 atoms of oxygen are utilized for each molecule of benzoic acid. The enzymes concerned are adaptive, and are rapidly formed when small amounts of benzoic acid are added to suspensions of the bacteria (2). Benzoic acid may be considered somewhat toxic to the bacteria, because, although small amounts stimulate enzyme formation, larger amounts slow down or inhibit the process, possibly by interfering with reactions providing the energy for the enzyme synthesis. Furthermore, as shown below, the enzyme once formed disappears fairly rapidly in the absence of benzoic acid. Streptomycin prevents the formation of these adaptive enzymes, and thus inhibits oxidation of benzoic acid (2). The rate of oxygen uptake has been carefully measured and the following is an analysis of the kinetics of the reactions involved. Data were obtained from experiments done with *Mycobacterium tuberculosis* BCG 8240. The technique has already been described (1, 2).

Oxidation of benzoic acid by *Mycobacteria* proceeds by a number of stages. The substances formed, for the most part, have not been isolated, and will be referred to by letters. Benzoic acid (A) is first hydrated (*cf.* (3)) to form B. B takes up 1 atom of oxygen and becomes C. C, in turn, takes up a second atom of oxygen to become D, and so on through E, F, and G, until 5 atoms of oxygen have been taken up for each molecule of the original benzoic acid. The time course of each of these reactions, since there is no change in the concentrations of either water or oxygen, will probably be monomolecular. Using small letters to mean "concentration of," we may express these rates as follows:

$$\frac{da}{dt} = -k_1a$$

$$\frac{db}{dt} = k_1a - k_2b$$

$$\frac{dc}{dt} = k_2b - k_3c$$

……

$$\frac{df}{dt} = k_5e - k_6f$$

Because we do not know how G is further transformed by the bacteria, except that it is not oxidized, it is difficult to write an equation for it; later it will be seen that it is unnecessary to do so. In each case the velocity constant is proportional to the amount of the corresponding enzyme present in any particular experiment; an increase in the constant implies an increase in the amount of enzyme.

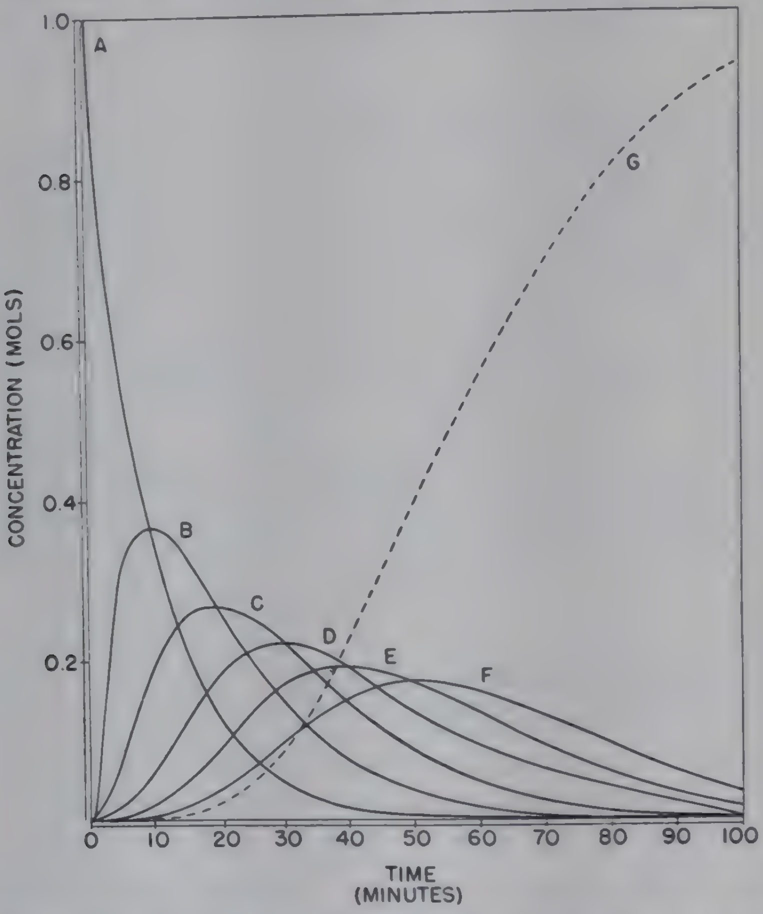


FIG. 1. The different curves give the amounts of benzoic acid (A) and substances B, C, D, E, and F at the times indicated. The original amount of A is taken as 1.0 mole and the k values are put equal to 0.1 each.

Equations similar to these have been integrated by Bateman (4). We may, however, begin by making the simplifying assumption that all the constants, k_1, k_2, etc., are equal. Integration then leads to a series of equations giving the amounts of each substance present at various times. The general solution giving the amount of the Nth substance is

$$n = e^{-kt} \frac{c_1}{(N-1)!}(kt)^{N-1} + \frac{c_2}{(N-2)!}(kt)^{N-2} + \dots c_{N+1}$$

The constants of integration, c_1, c_2, etc., may be evaluated by setting the condition that at zero time there is present in the mixture a_0 of A, and none of the other substances, B, C, etc. Then c_1 becomes a_0, and the other constants, c_2, c_3, etc., become zero. Fig. 1 shows the amounts of these substances present at various times when a_0 is given the value 1, and all the k values are put equal to 0.1.

To compute the oxygen consumption we proceed as follows. At the time t, each molecule of C that is present has taken up 1 atom of oxygen, each molecule of D has taken up 2 atoms, etc. G presents a slight difficulty since it may have been further transformed; we must therefore say that 5 atoms of oxygen have been taken up by all of the original benzoic acid molecules that have reached this stage or gone beyond it. But all molecules not in the forms A, B, C, D, E, or F have reached this stage or gone beyond it, and their concentration must therefore be $a_0 - (a + b + c + d + e + f)$. The total oxygen taken up, X, will then be given by

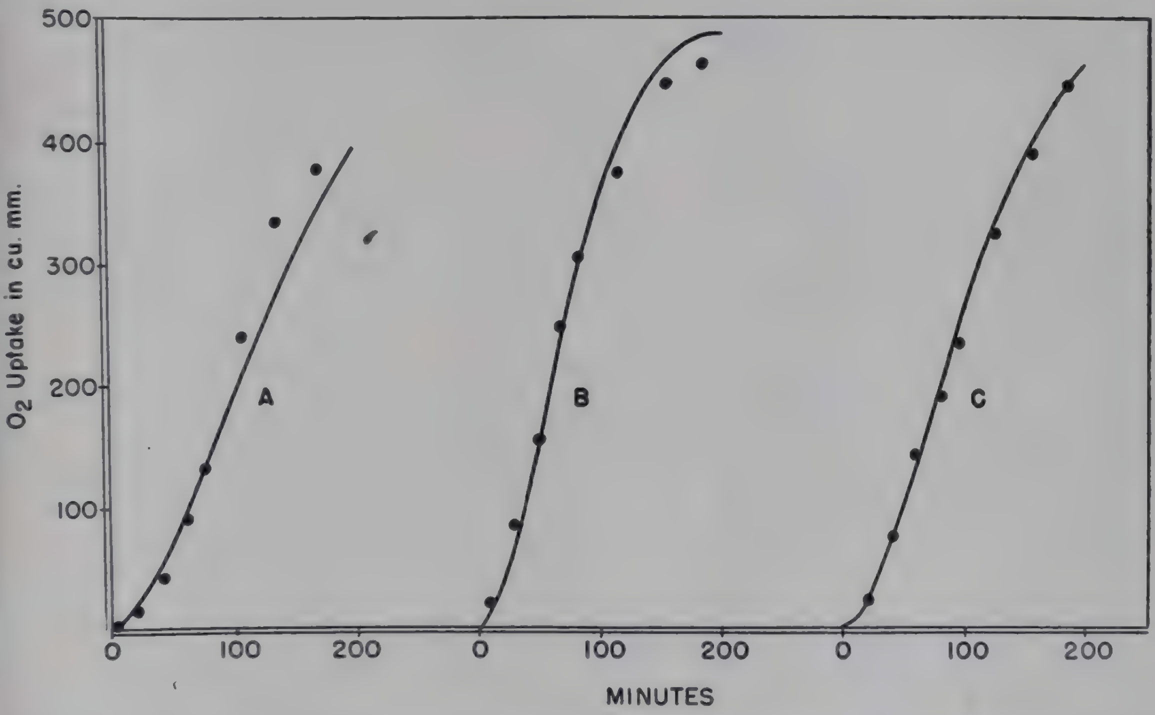


FIG. 2. Oxidation of 1.0 mg. of benzoic acid by *Mycobacterium tuberculosis* BCG 8240 preincubated for 90 minutes (Curve A) without benzoic acid; Curve B and C with 0.05 mg. of benzoic acid. 1.0 mg. of benzoic acid was added to each after preincubation, and in addition 10 γ of streptomycin (Curve C). The points are experimental, and the curves are theoretical; computed for Curve A $k = 0.028$, Curve B $k = 0.050$, and Curve C $k = 0.038$.

$$X = c + 2d + 3e + 4f + 5(a_0 - (a + b + c + d + e + f))$$

which simplifies to

$$X = 5a_0 - (5a + 5b + 4c + 3d + 2e + f)$$

When appropriate values for the constants a_0 and k are chosen, oxygen consumption at various times may be computed; the smooth curves in Fig. 2 were constructed from such computations. Experimental data, adjusted so that a_0 corresponds to exactly 500 c.mm. of oxygen, were plotted on these theoretical curves. It was then seen that the experimental points, although forming a curve of precisely the same shape as the theoretical, all lay a short distance to the right of it. When shifted 10 to 15 minutes to the left, the fit was excellent, as shown in Fig. 2. This shift implies a latent period before any great amount of oxidation occurs; this time interval is presumably occupied by formation of enzyme. It will be discussed further below.

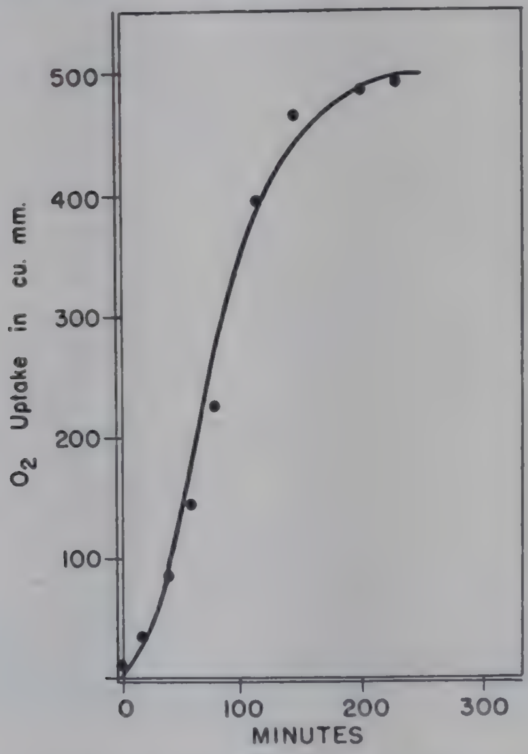


FIG. 3. A smooth curve is made up on the assumption that the velocities of the oxidations of E to F and of F to G are increased so that E and F do not accumulate in any significant amount. The points are experimental.

Of five experiments analyzed, three yielded results that fitted such curves; two did not. In these cases the assumption was then made that the velocities of the oxidations of E to F and of F to G were increased to such an extent that these substances, F and G, never accumulated in any significant amount. New curves based on this additional assumption were constructed, and experimental results were found to fit these in a satisfactory manner (Fig. 3).

Table I gives values for the k constants and the lengths of the latent periods for one set of experiments. For convenience a set was chosen in which the k values were equal in each experiment. It will be seen that the latent period is longest when no benzoic acid was used for preincubation; it disappears completely after 90 minutes preincubation with 0.2 mg. of benzoic acid. These facts may be explained on the assumption already mentioned, that benzoic acid has a double action. In very low concentrations it stimulates the formation of the oxidizing enzymes, while in high concentrations its toxic action tends to depress the formation of enzymes. Thus when no benzoic acid was added during preincubation no enzymes were formed, the latent period was long, and the total enzyme formed was least, as shown by the value for k. With very small amounts, some enzyme was formed, but the addition of the large amount of benzoic acid for the experiment induced some further formation. The latent period was shorter. With larger amounts the maximum amount of enzyme is formed, the k reaches a maximum, and the latent period vanishes. Enzyme already formed is apparently not interfered with.

TABLE I

Values for k Constants and Lengths of Latent Periods

Experiment No.	Benzoic acid used for preincubation	Without streptomycin		With streptomycin
		Latent period	k	k
	mg.	*min.*		
13-14	None	18	0.028	0.017
16-17	0.05	10	0.050	0.038
21-22	0.10	4	0.050	0.050
24-25	0.20	0	0.050	0.050

TABLE II

Demonstration of Disappearance of Enzyme

In this experiment k_5 and k_6 were larger than the others; the values given are for k_1 to k_4 which were equal.

Time of preincubation with 10 γ benzoic acid	k
min.	
0	0.43
60	0.67
90	0.67
120	0.50
240	0.36

Disappearance of enzyme was demonstrated in the following way. Bacteria was preincubated with 0.01 mg. of benzoic acid for various lengths of time. As long as benzoic acid was present the enzymes increased to a maximum; after it was exhausted they gradually decreased. Data are given in Table II.

The effect of streptomycin is to decrease the velocities of all the reactions equally; there is no specific effect on any particular stage. This is true whether all the k values are equal, as in Table I, or whether they are not, as in other experiments.

SUMMARY

The kinetics of the oxidation of benzoic acid by *Mycobacterium tuberculosis* BCG 8240 have been analyzed, and six consecutive monomolecular reactions have been postulated. In most cases the velocity constants for these reactions were the same; in other cases those for reactions 5 and 6 were considerably greater, due to a relative increase in the amounts of the adaptive enzymes which catalyze these processes. Oxidation begins with a latent period during which adaptive enzymes are formed. If the bacteria are previously treated with small amounts of benzoic acid, the latent period becomes shorter or vanishes, and the constants become greater up to a maximum, indicating the presence of increased amounts of enzyme. Enzymes gradually disappear in the absence of benzoic acid.

The effect of streptomycin is to depress equally all stages of oxidation.

Our thanks are due to Dr. J. M. Thomas of the Department of Mathematics for help in computing oxygen consumption curves.

BIBLIOGRAPHY

1. Fitzgerald, R. J., and Bernheim, F., *J. Bact.*, **54**, 671 (1947).
2. Fitzgerald, R. J., Bernheim, F., and Fitzgerald, D. B., *J. Biol. Chem.*, **175**, 195 (1948).
3. Stanier, R. Y., *J. Bact.*, **55**, 477 (1948).
4. Bateman, H., *Proc. Cambridge Phil. Soc.*, **15**, 423 (1908–10).

THE PRODUCTS OF PROTEOLYSIS OF SOME PURIFIED PROTEINS*

By ANNE BELOFF and CHRISTIAN B. ANFINSEN

(*From the Department of Biological Chemistry, Harvard Medical School, Boston*)

(Received for publication, July 30, 1948)

Recent studies on the digestion of proteins by the proteolytic enzymes of the gastrointestinal tract have resulted in a considerable revision of our concepts on how these enzymes act.

Tiselius and Eriksson-Quensel (1) studied the mechanism of the peptic digestion of crystalline egg albumin by electrophoretic examination of the products of proteolysis. They observed that, as digestion proceeded, the average size of the peptides formed remained constant. From these results, they postulated that each protein molecule is rapidly degraded to its ultimate products without the intervening production of larger units. They have called this an "all or none" mechanism of proteolysis. Haugaard and Roberts (2) reached similar conclusions from experiments on the digestion of β-lactoglobulin by crystalline pepsin. They reported that during digestion the increase in terminal amino nitrogen (determined by the nitrous acid manometric technique of Van Slyke (3)) increased lineally with the total non-protein nitrogen. A similar study was made by Winnick (4) on the digestion of casein by chymotrypsin, trypsin, pepsin, ficin, and papain. His data were consistent with those of the above workers.

We have extended these observations in the present paper, employing for our studies trypsin acting on crystalline bovine serum albumin and purified γ-globulin, and pepsin on crystalline bovine serum albumin, purified bovine fibrin, and twice recrystallized egg albumin. The results to be presented also favor a rapid or immediate degradation of the protein molecules to peptides of characteristic size, with the liberation of little or no free amino acid nitrogen.

EXPERIMENTAL

Enzymes and Substrates—Solutions of crystalline trypsin were made in 0.05 M phosphate buffer at pH 7.8. The pepsin was suspended in water and dialyzed free of $MgSO_4$ before each experiment.

The substrates were dissolved in 0.05 M phosphate buffer, pH 7.8, for the trypsin experiments and in HCl at pH 1.8 for peptic digestion.

---

* This work was supported in part by a grant-in-aid from the Josiah Macy, Jr., Foundation.

We are indebted to the Department of Physical Chemistry of the Harvard Medical School for our supply of protein substrates.[1] Fibrin was prepared by clotting the fibrinogen in Fraction I of bovine serum (7) with thrombin.

For experiments with fibrin, the substrate was suspended in HCl or homogenized in HCl at pH 1.8.

Procedure—The solutions of protein were incubated with the enzyme at 38° in the amounts indicated in Tables I to V. Digestion was carried out in 50 cc. Erlenmeyer flasks, and suitable aliquots were withdrawn at given time intervals for analysis. In the experiments with pepsin, in which the digestion products were separated by dialysis (see below), the substrates were incubated in a cellophane dialyzing sac and dialyzed against an equal volume of HCl at pH 1.8.

Method of Analysis—Three different procedures were employed for the removal of undigested protein from the samples to be analyzed.

In the first, the protein was precipitated with an equal volume of 10 per cent trichloroacetic acid or 1 per cent picric acid. The solution was filtered, and in the case of trichloroacetic acid, the filtrates were acidified to pH 1 with HCl and extracted with ether. This extraction was necessary, as the trichloroacetic acid interfered with the subsequent analysis; it was omitted on picric acid filtrates. All experiments with trypsin were carried out by this procedure.

In the second method, digestion was carried out in a cellophane sac suspended in HCl at pH 1.8. Analyses were then made on aliquots of the dialysates.

Finally, experiments were carried out with a rocker-perfusion apparatus originally designed for cultivation of malarial parasites (8). In this method, the products of proteolysis were continuously removed by dialysis against a stream of aqueous HCl, pH 1.8, passing through a cellophane coil immersed in the digestion mixture.

Aliquots of the protein-free filtrates and dialysates, prepared as described above, were analyzed for terminal amino nitrogen by the Van Slyke nitrous acid method (3) and for free amino acid nitrogen by the ninhydrin method (9). The amino acids bound in peptides were determined by the ninhydrin method after hydrolysis. This is referred to in Tables I to V as total amino nitrogen.

---

[1] The products of the bovine plasma fractionation employed in this work were prepared by the Armour Laboratories, Chicago, by the method developed by the Department of Physical Chemistry, Harvard Medical School ((5, 6) and Oncley, J. L., Melin, M., Richert, D. A., Cameron, J. W., and Gross, P. M., Jr., in preparation) under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. The blood for human plasma fractionation was collected by the American Red Cross.

The average number of amino acid residues per peptide molecule was determined by the ratio of the "total" to the "terminal" amino nitrogen. A correction was made for free amino acid nitrogen when significant. All values are expressed as mg. of NH_2-N or COOH-N released in the total reaction mixture.

Hydrolysis—The peptides were hydrolyzed in 6 N HCl at 110° for 12 to 18 hours. The hydrolysates were concentrated to dryness *in vacuo* over sodium hydroxide pellets to remove excess HCl, and the residues were made up to a known volume for analysis.

TABLE I

Digestion of Bovine Serum Albumin and Human γ-Globulin by Crystalline Trypsin at pH 7.8

Experiment 1, 100 cc. of 2 per cent serum albumin + 20 mg. of trypsin; Experiment 2, 100 cc. of 2 per cent serum albumin + 50 mg. of trypsin; Experiment 3, 50 cc. of 1 per cent γ-globulin + 20 mg. of trypsin.

Experiment No.	Time of sampling	Free amino acid nitrogen (COOH-N)	Terminal amino nitrogen (NH_2-N)	Total amino nitrogen (COOH-N)	$\frac{\text{COOH-N}}{NH_2\text{-N}}$, average No. of amino acid residues per peptide molecule
	hrs.	*mg.*	*mg.*	*mg.*	
1	4	Not analyzed	5.1	14	2.7
	8	" "	8.4	22	2.6
	24	0.6	13.5	42	3.2
	48	1.4	23.5	60	2.7
2	34		13.5	35	2.6
	48		13.8	36	2.7
	56		13.8		
3	4		0.75	2.0	2.7
	9		1.30	3.5	2.7
	24	<1% terminal amino-N	1.75	5.6	3.2
	48	<1% " "	2.34	8.5	3.6

Results

Digestion of Serum Albumin and γ-Globulin with Trypsin—The results of experiments with trypsin are given in Table I. Increasing the concentration of trypsin from 20 to 50 mg. did not accelerate digestion. It is evident that the digestion of γ-globulin by trypsin proceeds very slowly, but it is of interest that there is no evidence for the presence of larger peptides in the protein-free filtrate, although the first analyses must represent the early stages of digestion. For serum albumin, the average number of amino acid residues per peptide molecule is about 2.7; for γ-globulin, the values ranged from 2.7 to 3.6.

Digestion of Crystalline Egg Albumin and Serum Albumin with Pepsin—Owing to the slow rate of digestion of native proteins by trypsin, the following experiments were carried out with crystalline pepsin. Table II gives the results of the digestion of egg albumin and serum albumin by this enzyme.

In Experiment 1, the undigested egg albumin was precipitated with 10 per cent trichloroacetic acid as previously described. In Experiment 2, the undigested serum albumin was precipitated with picric acid. It is evident from Experiment 1 that, although after 4 hours the digestion was nearing completion, the subsequent 20 hour incubation of the peptides with the enzyme resulted in no further degradation of the peptide molecules.

TABLE II

Digestion of Egg Albumin and Bovine Serum Albumin by Crystalline Pepsin at pH 1.8

Experiment 1, 100 cc. of 1 per cent egg albumin + 10 mg. of pepsin; Experiment 2, 15 cc. of 3.3 per cent serum albumin + 10 mg. of pepsin.

Experiment No.	Time of sampling	Free amino acid nitrogen (COOH-N)	Terminal amino nitrogen (NH_2-N)	Total amino nitrogen (COOH-N)	$\frac{\text{COOH-N}}{\text{NH}_2\text{-N}}$, average No. of amino acid residues per peptide molecule
	hrs.	*mg.*	*mg.*	*mg.*	
1	4		10.7	72.0	6.7
	6		11.4		
	24	0.32	13.2	93.0	7.0
2	0.25		1.2	4.4	3.7
	4	<1% terminal amino N	3.2	10.1	3.2

These experiments on egg albumin, in which the value of seven amino acid residues per peptide molecule was obtained, are in good agreement with the value obtained by Tiselius and Eriksson-Quensel who found the average molecular weight of the peptides to be 1080 from diffusion coefficient measurements. For serum albumin, the average ratio of the total peptide bond amino nitrogen to terminal amino nitrogen was about 3.5, or about half that of egg albumin.

Digestion of Purified Fibrin with Pepsin—Fibrin was studied as an example of a fibrous protein. In preliminary experiments, the protein was suspended in HCl and digestion carried out in a cellophane dialyzing sac as described above. The results of two such experiments are given in Table III, Experiments 1 and 2, in which the average number of amino acid residues per peptide molecule varied over a range of 3.3 to 5.1 In some other experiments, there was an even greater variation in the ratio of the total amino nitrogen to the terminal amino nitrogen at different times during digestion (in the range of 3 to 7), and this did not appear to be a function of the time of incubation. This variation may be due to the fact that the fibrin forms a gelatinous mass when suspended in HCl. More consistent results were obtained by preparing a homogenate of fibrin in HCl at pH 1.8, before the addition of the enzyme. The results of two such experiments are given in Table III, Experiments 3 and 4, and show a variation in the ratios from 3.7 to 4.4. The particular interest of these experiments on fibrin is the relatively high concentration of free amino acid liberated, most of which occurs in the early stages of digestion. In the experiments reported in Table III, values from 11 to 28 per cent of the total amino nitrogen were present as free amino acid nitrogen after the first 15 to 20 minutes of digestion. After 1 hour, the rate of increase of free amino acid nitrogen was small as compared to that of the terminal peptide amino nitrogen. The possible release of non-protein amino nitrogen by homogenizing the fibrin was controlled by analyzing the dialysate of a sample of homogenate incubated without the addition of enzyme. No amino nitrogen was detected after 3.5 hours of incubation.

TABLE III

Digestion of Purified Bovine Fibrin by Crystalline Pepsin at pH 1.8

Experiment 1, 350 mg. of fibrin suspended in 20 cc. of HCl + 50 mg. of pepsin; Experiment 2, 250 mg. of fibrin suspended in 5 cc. of HCl + 50 mg. of pepsin; Experiment 3, 200 mg. of fibrin homogenized in 10 cc. of HCl + 10 mg. of pepsin; Experiment 4, 200 mg. of fibrin homogenized in 10 cc. of HCl + 10 mg. of pepsin.

Experiment No.	Time of sampling	Free amino acid nitrogen (COOH-N)	Terminal amino nitrogen (NH_2-N)	Per cent of NH_2-N as free amino acid	Total amino nitrogen (COOH-N)	$\frac{\text{COOH-N}}{\text{NH}_2\text{-N}}$, average No. of amino acid residues per peptide molecule (corrected for free amino acids)
	hrs.	*mg.*	*mg.*		*mg.*	
1	0.5	0.26	0.91	28	2.42	3.3
	1	0.28	2.48	11	7.7	3.3
	4	0.28	8.90	3	33.8	3.9
2	0.25	0.02	0.11	22	0.39	4.1
	0.75	0.10	0.74	13	2.50	3.7
	2	0.21	2.58	8	11.80	4.9
	3.5	0.24	4.20	6	20.5	5.1
3	0.3	0.07	0.38	17	1.31	4.0
	1	0.13	1.68	8	6.03	3.8
	3.5	0.24	3.09	8	11.05	3.8
4	0.5	0.07	0.69	11	2.38	3.7
	3	0.14	1.94	7	8.16	4.4

Removal of Digestion Products by Dialysis—The evidence that has been presented by Bergmann and his colleagues on enzymatic synthesis of peptide bonds, and also on the effect of peptides acting as "cosubstrates," has frequently been advanced as an objection to the study of the peptides formed by enzymatic hydrolysis of proteins in relation to the protein structure or to the enzyme specificity.

One method of reducing the likelihood of these secondary reactions occurring is to remove the peptides by dialysis as they are formed during digestion. The rocker-perfusion method referred to in the experimental section was used for this purpose. The results of such an experiment, with serum albumin as substrate, are given in Table IV. The average value of the number of amino acid residues per peptide molecule is not significantly different from the results found without dialysis, giving an average of 3.6 (Table II, Experiment 2). It is seen from these results that the amino nitrogen value in the first 15 minute dialysate is low (due to dilution by the HCl initially present in the dialyzing tube). In the following 3 hours, the total amino nitrogen found in the dialysate per unit time remained relatively constant.

TABLE IV

Digestion of Bovine Serum Albumin by Pepsin in Which Peptides Are Removed by Dialysis

10 cc. of 5 per cent serum albumin + 10 mg. of pepsin.

Period of collection of dialysate	Terminal amino nitrogen (NH_2-N)	Total amino nitrogen (COOH-N)	$\frac{\text{COOH-N}}{\text{NH}_2\text{-N}}$, average No. of amino acid residues per peptide molecule
hrs.	*mg.*	*mg.*	
0 –0.25	0.15	0.45	2.9
0.25–0.75	0.79	3.04	3.8
0.75–1.25	0.85	3.05	3.6
1.25–1.75	0.74		
1.75–2.25	0.60	2.28	3.8
2.25–3.25	1.12		
3.25–4.25	0.86	2.82	3.3
4.25–5.25	0.57	2.02	3.5
5.25–6.25	0.59		
6.25–7.25	0.44	1.95	4.4
7.25–8.25	0.37		
Average			3.6

One cannot ascertain from this experiment whether the rate of digestion or the rate of dialysis is the limiting factor. If the rate of dialysis is slow in relation to the rate of proteolysis, a high concentration of peptides will accumulate in the digestion vessel.

In Experiment 1, Table V, analyses were made at given times on the peptides in the dialysate, Solution A, and also in the dialyzed solution,

TABLE V

Digestion of Bovine Serum Albumin by Crystalline Pepsin with and without Removal of Peptides by Dialysis

Experiment 1, 15 cc. of 3.3 per cent serum albumin + 10 mg. of pepsin; Solution A, dialysate; Solution B, dialyzed solution; Solution C, closed system of digestion. Experiment 2, 15 cc. of 3.3 per cent serum albumin + 2 mg. of pepsin.

Experiment No.	Solution	Period of collection of dialysate and time of sampling	Terminal amino nitrogen (NH_2-N)	Total amino nitrogen (COOH-N)	$\frac{\text{COOH-N}}{\text{NH}_2\text{-N}}$, average No. of amino acid residues per peptide molecule
		hrs.	*mg.*	*mg.*	
1	A	0–1	0.93	3.94	4.2
		1–2	1.12	4.50	4.0
		2–3	0.89	3.22	3.7
		3–4	0.65	2.29	3.5
		4–5	0.41	1.52	3.6
Average.					3.8
	B	0–0.5	7.46	24.8	3.3
		0.5–1	7.30	22.8	3.0
		1–2	5.67	20.8	3.7
		2–3	4.08	11.9	3.0
		3–4	3.82	16.5	4.3
		4–5	2.50	10.7	4.3
Average.					3.6
	C	0–0.5	5.58	17.6	3.2
		0.5–1	5.83	18.7	3.2
		1–2	6.15	21.1	3.2
		2–3	6.73	21.4	3.1
		3–4	8.3	23.6	2.9
Average.					3.1
2		0–0.25	0.09	0.37	4.1
		0.25–0.5	0.35	1.34	3.8
		0.5–1	0.56	1.86	3.3
		1–2	1.17	3.38	2.9
Average.					3.5

Solution B. Simultaneously, a digestion system was set up in a closed vessel, Solution C (as used in all earlier experiments). 2 cc. aliquots of Solutions B and C were withdrawn at the times indicated and neutralized to stop digestion. These aliquots were then dialyzed against 10 cc. of water at 4° overnight. In this procedure, the use of a protein precipitant was omitted.

The results indicate that there is no significant difference in the peptide lengths obtained whether or not dialysis is employed. However, this experiment also shows that the rate of dialysis is inadequate for the complete removal of peptides as they are formed, since the dialyzed amino nitrogen values are much less than the corresponding values analyzed in the dialyzed digestion mixture. In order to decrease this accumulation of peptides, a similar system of dialysis as described above was used, but only 2 mg. of pepsin were added. The results are given in Table V, Experiment 2. The results show no significant difference from those reported in Experiment 1.

DISCUSSION

In the experiments reported here, we have not observed any significant variation in the size of the peptides formed during digestion. This is consistent with the "all or none" hypothesis advanced by Tiselius and Eriksson-Quensel discussed above. Although the evidence indicates that there is not a gradual degradation of the protein molecule, it cannot be overlooked that our analyses were performed on trichloroacetic acid or picric acid filtrates or the dialyzable fractions. Therefore, large molecules resulting from partial degradation could be present in the protein precipitate or in the non-dialyzable fraction.

The work of Petermann (10) suggests an alternate mechanism of proteolysis. From ultracentrifuge studies of the peptic digestion products of beef serum pseudoglobulin, at pH values from 2.7 to 4.5, she was able to demonstrate the presence of components of high molecular weight (probably halves and quarters of the protein molecule) under the more alkaline conditions. She suggests that at the optimum pH for enzymatic activity the breakdown may occur too rapidly to observe intermediate components. Bridgman (11), studying the peptic digestion of human γ-globulin by ultracentrifuge analysis, also identified half molecules of the protein. He obtained a maximum yield of the latter at pH 3.5.

The differences between these findings and our own and those of other workers may be due to the fact that proteolysis varies, depending on the structure of the protein substrate. It is of interest that fibrin (the only fibrous protein studied so far in relation to the mechanism of proteolysis) should behave differently from the other substrates in that it gives a greater release of free amino acids, and that this occurs in the initial stages of digestion.

In recent years, considerable work has been done on the partial acid hydrolysis products of proteins in relation to protein structure. There has been very little effort, however, to apply enzymatic hydrolysis to such studies, although it is probable that one could get easily reproducible results by this method. The objections to the application of enzymatic hydrolysis in these studies has been discussed above, and it is felt that the use of some method for the continual removal of peptides from the digestion mixture as described in this paper may be of value in future development of work on these lines.

The work on the specificity of proteolytic enzymes by Bergmann and his colleagues has been applied to synthetic substrates. From these findings, the conception has arisen that the proteolytic enzymes have very restricted specificity. According to present experiments, however, as much as 30 per cent of the peptide bonds of serum albumin is hydrolyzed by pepsin. Therefore, a further application of the study of proteolysis may be made in relation to enzyme specificity by a study of the amino acid composition of some of the peptides formed.

SUMMARY

1. The digestion of crystalline bovine serum albumin and human γ-globulin by crystalline trypsin and of crystalline bovine serum albumin, purified fibrin, and twice recrystallized egg albumin by crystalline pepsin has been studied.
2. The average number of amino acid residues per peptide molecule formed during proteolysis was estimated. These values remained approximately constant throughout digestion. The significance of this finding in relation to the enzymatic mechanism is discussed.
3. The free amino acids liberated were also analyzed. Peptic digestion of fibrin showed a comparatively high concentration of free amino acids released in the first 30 minutes of digestion.
4. A system for the continual removal of the peptides from the digestion mixture by dialysis is described.
5. The possible applications of studies on the products of proteolysis of purified proteins are discussed.

BIBLIOGRAPHY

1. Tiselius, A., and Eriksson-Quensel, I., *Biochem. J.*, **33**, 1752 (1939).
2. Haugaard, G., and Roberts, R. M., *J. Am. Chem. Soc.*, **64**, 2664 (1942).
3. Van Slyke, D. D., *J. Biol. Chem.*, **83**, 425 (1929).
4. Winnick, T., *J. Biol. Chem.*, **152**, 465 (1944).
5. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, **68**, 459 (1946).
6. Cohn, E. J., Hughes, W. L., Jr., and Weare, J. H., *J. Am. Chem. Soc.*, **69**, 1753 (1947).

7. Edsall, J. T., Ferry, R. M., and Armstrong, S. H., Jr., *J. Clin. Invest.*, **23**, 557 (1944).
8. McKee, R. W., Ormsbee, R. A., Anfinsen, C. B., Geiman, Q. M., and Ball, E. G., *J. Exp. Med.*, **84**, 569 (1946).
9. Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 627 (1941).
10. Petermann, M., *J. Phys. Chem.*, **46**, 183 (1942).
11. Bridgman, W. B., *J. Am. Chem. Soc.*, **68**, 857 (1946).

A REINVESTIGATION OF FLAVACIDIN, THE PENICILLIN PRODUCED BY ASPERGILLUS FLAVUS

By M. ADLER and O. WINTERSTEINER

(*From the Division of Organic Chemistry, The Squibb Institute for Medical Research, New Brunswick*)

(Received for publication, July 20, 1948)

In a preliminary communication Fried, Koerber, and Wintersteiner (1) reported the results of their study in 1944 on the chemical nature of "flavacidin," the penicillin produced by *Aspergillus flavus*. The evidence then available seemed to indicate that flavacidin was a new penicillin not produced by *Penicillium notatum*; namely, 3-pentenylpenicillin, mixed with some benzylpenicillin (penicillin G).[1]

The tentative identification of the main component as 3-pentenylpenicillin was based on the following findings (3). Though the analyses of the crystalline sodium salt of flavacidin were not quite conclusive, they favored on the whole a composition $C_{14}H_{17-19}O_4N_2SNa$. The formula with 19 H atoms is that of the sodium salt of 2-pentenylpenicillin, R = $CH_3\cdot CH_2\cdot CH{=}CH\cdot CH_2^-$, one of the entities produced by *Penicillium notatum*, but a comparison of the x-ray diffraction pattern of a specimen of the latter compound, previously isolated at the Squibb Institute, with that of the flavacidin salt revealed such marked differences as to preclude identity with this penicillin. The flavacidin salt was then degraded to the penilloaldehyde, $R\cdot CO\cdot NH\cdot CH_2\cdot CHO$ (I), by acid hydrolysis and subsequent treatment with mercuric chloride (4). The penilloaldehyde was isolated as the 2,4-dinitrophenylhydrazone. The analysis of this derivative conformed with the calculated values for the dinitrophenylhydrazone of a pentenylpenilloaldehyde (I, R = C_5H_9), but it strongly depressed the melting point of a penilloaldehyde dinitrophenylhydrazone which had been previously obtained in the same manner by degradation of crystalline sodium 2-pentenylpenicillinate from *Penicillium notatum* (5). Moreover, the x-ray diffraction patterns[2] of the two degradation products were unmistakably different, and this was also the case when the dinitrophenylhydrazone of synthetic 3-hexenoylaminoacetaldehyde (I, R = $CH_3 \cdot CH_2 \cdot CH{=}CH \cdot CH_2-$) was substituted for that derived from 2-pentenylpenicillin by degradation. On the other hand, the penilloaldehyde dinitrophenylhydrazone from flavacidin and the derivatives obtained from synthetic 4-hexenoylaminoacetaldehyde (I, R = $CH_3 \cdot CH{=}CH \cdot CH_2 \cdot CH_2-$) showed close correspondence of the patterns, indicating that the precursor of the former was 3-pentenylpenicillin. It was realized that this conclusion would have to be substantiated by rigid characterization of the acid $R \cdot COOH$, but the amounts of crystalline salt obtained in 1944 did not suffice for further degradation work.

---

[1] The nomenclature employed here conforms with that adopted in the forthcoming monograph, "The chemistry of penicillin," (2) and is based on the R group differentiating the various penicillins instead of on the arbitrary designation by letters or numbers hitherto used. In the new terminology, penicillin F is 2-pentenylpenicillin; dihydropenicillin F, *n*-amylpenicillin; penicillin G, benzylpenicillin; penicillin K, *n*-heptylpenicillin; and penicillin X, *p*-hydroxybenzylpenicillin.

[2] Measurements by N. C. Schieltz, Northern Regional Research Laboratory, United States Department of Agriculture, Peoria, Illinois; private communication, June 8, 1944.

The study was resumed in 1946 when a larger batch of fermentation broth from *Aspergillus flavus* was prepared in the Squibb penicillin plant. For the brown sugar-containing medium employed in the original work, one containing lactose was substituted, a measure which raised the potency of the broth from about 20 units to 70 units per cc. The culture filtrate was worked up in the same manner as the 1944 batch; that is, by the usual multiple solvent extraction procedure, except that some further fractionation was accomplished in the last step by conducting the extraction of the final ether solution with sodium bicarbonate in three stages, corresponding to a final pH of the resulting sodium salt solution of 6.0, 6.5, and 7.2, respectively. Exploratory chromatographic and crystallization experiments showed that only the first of these fractions, which represented 62 per cent of the total units recovered as sodium salts, readily yielded crystalline material by the isolation procedure used in 1944. However, it soon became clear from analytical and ultraviolet absorption data that this crystalline product differed from that obtained in 1944 in that it contained preponderantly benzylpenicillin instead of a penicillin of the F type. After purification by chromatographing on alumina, as in the isolation of benzylpenicillin (6) and of flavacidin (2), the benzylpenicillin was removed by the triethylamine procedure (7). The material not precipitable by triethylamine was converted into a crystalline ammonium salt, which was spectrographically free from benzylpenicillin (absence of phenyl bands in the range 2500 to 2700 A). The analyses of this product, and those of a potassium salt prepared from it, indicated that we were dealing with an amylpenicillin rather than a pentenylpenicillin. The Craig distribution curve (8) of the potassium salt (Fig. 1) showed but one maximum, but its shape, and the trends in the calculated distribution coefficients (9) and in the *Bacillus brevis-Staphylococcus aureus* assay ratios over the range of the curve definitely indicated inhomogeneity. A sample shaken with hydrogen in the presence of platinum oxide, conditions under which 2-pentenylpenicillin is readily reduced to *n*-amylpenicillin (10), took up in sluggish reaction about 20 per cent of the hydrogen volume required by an ammonium pentenylpenicillinate. On the assumption that the original product consisted mainly of the *n*-amylpenicillin mixed with a much lesser amount of a pentenylpenicillin, it was hydrolyzed with strong hydrochloric acid in order to secure and characterize the side chain fatty acids. The acidic fraction, which accounted for about 80 per cent of the ether-soluble products, consisted of substantially pure *n*-caproic acid as shown by analysis, physical properties, and conversion to the crystalline *p*-toluidide. There was no doubt, therefore, that the main component of the ammonium salt mixture was *n*-amylpenicillin and not a pentenylpenicillin. That the minor constituent revealed by counter-current distribution was a penicillin of the latter type could be surmised from the small but definite hydrogen uptake. The hexenoic acid derived from this entity was apparently converted, under the influence of the mineral acid used in the hydrolysis, into a lactone which remained in the neutral portion of the ether-soluble products, and was later lost by volatilization.

Next, the potassium salt, as well as the contents of Tubes 14 to 20 from the counter-current distribution experiment, which could be assumed to consist of substantially pure *n*-amylpenicillin, were degraded to the penilloaldehyde by the procedure mentioned earlier. The dinitrophenylhydrazones thus obtained were recrystallized till their melting points were constant (182.5° and 183.5°, respectively). As expected, neither preparation depressed the melting point (188°) of synthetic *n*-caproylaminoacetaldehyde dinitrophenylhydrazone. However, it was somewhat surprising to find that their mixtures with the penilloaldehyde dinitrophenylhydrazone obtained in 1944 (m.p. 182°) and with the sample of synthetic 4-hexenoylaminoacetaldehyde dinitrophenylhydrazone used for comparison at that time likewise showed no melting point depression. A new specimen of the latter compound was then synthesized and carefully purified. Its melting point was somewhat higher (186°) than that of the old preparation, but again no depression was observed in mixture, either with the present penilloaldehyde dinitrophenylhydrazone or with the synthetic *n*-caproyl derivative. In the hope that the x-ray diffraction patterns would reflect the structural differences between the two synthetic compounds and thus achieve the desired differentiation, the two penilloaldehyde dinitrophenylhydrazone preparations from the present batch and the two synthetic compounds were submitted to Dr. G. L. Clark of the Department of Chemistry, University of Illinois, who kindly consented to have the necessary measurements carried out in his laboratory.[3] However, the diffraction patterns of the four preparations were practically identical, nor could they be distinguished from the patterns obtained in 1944 in the Northern Regional Research Laboratory from the compounds then submitted (dinitrophenylhydrazones of the degradation product and of synthetic 4-hexenoylaminoacetaldehyde).

---

[3] We wish to express our sincerest thanks to Dr. Clark for his cooperation and the valuable assistance he thereby rendered in clarifying this problem.

It was clear, then, that the dinitrophenylhydrazones of *n*-caproyl- and 4-hexenoylaminoacetaldehyde cannot be distinguished by either melting point or x-ray data, and that consequently the flavacidin obtained in 1944 could have been either 3-pentenylpenicillin or *n*-amylpenicillin. However, since there is no other conclusive evidence for the existence of a 3-pentenylpenicillin, whereas *n*-amylpenicillin is known to be a metabolic product of another aspergillic species, namely *Aspergillus giganteus* (11), as well as of *Penicillium notatum* (12), it appears highly probable that the entity isolated in 1944 was actually the latter penicillin. The fact that the analytical composition of the penilloaldehyde dinitrophenylhydrazone then obtained corresponded to the formula with 2 hydrogen atoms less was probably due to contamination with some of the phenylacetylaminoacetaldehyde derivative, originating in the small amount of benzylpenicillin in the flavacidin sodium salt.

The remainder of the study was concerned with adducing evidence for the nature of the R group in the minor component, presumably a pentenylpenicillin, present in the benzylpenicillin-free crystalline preparations. To attempt the isolation of this entity in form of a pure salt seemed impractical in view of the small proportion present and the difficulties attending the separation of such closely related penicillins. Similar difficulties could be anticipated in the separation of small amounts of the derived fatty acids; moreover, the danger of double bond shifts in hexenoic acids or of their conversion to lactones by the vigorous hydrolytic treatment necessary for their liberation could not be disregarded. We therefore resorted to an indirect but milder method, in which the penilloaldehyde dinitrophenylhydrazone mixture was used as the starting material, and which is exemplified below for 4-hexenoylaminoacetaldehyde dinitrophenylhydrazone.

$$CH_3CH{:}CH\cdot CH_2\cdot CH_2\cdot CO\cdot NH\cdot CH_2\cdot CH{:}N\cdot NH\cdot C_6H_3\cdot (NO_2)_2 \xrightarrow{OsO_4}$$

$$[CH_3CHOH\cdot CHOH\cdot CH_2\cdot CH_2\cdot CO\cdot NH\cdot] \xrightarrow{HIO_4}$$

$CH_3\cdot CHO$ (isolated as dinitrophenylhydrazone)

To make sure that these reactions proceeded in the expected fashion, 4-hexenoic acid *p*-toluidide was treated with osmic acid in ether. The yield of pure 4,5-dihydroxycaproic acid *p*-toluidide was 46 per cent. Oxidation of the latter compound with periodic acid gave acetaldehyde, which was isolated as the 2,4-dinitrophenylhydrazone. The weight of the derivative corresponded to 65 per cent of the calculated amount. Similar experiments, but without isolation and characterization of the intermediate glycols, were performed with 4-hexenoic acid itself and with 4-hexenoylaminoacetaldehyde dinitrophenylhydrazone. In the latter case the over-all yield of acetaldehyde was 48 per cent of the theoretical, showing that the side products which were undoubtedly found in both oxidation steps did not interfere with the isolation of the volatile aldehydic fragment. The choice of the penilloaldehyde dinitrophenylhydrazone as the starting product for the oxidative degradation was dictated by the necessity, on the one hand, of avoiding the complications to be expected from the presence of sulfur-containing products derived from the penicillamine moiety, and, on the other hand, of securing the moiety containing the R group in the form of a derivative which could be isolated in reasonably good yield, thus minimizing the danger of losing by fractionation a substantial part of the unsaturated component of the mixture.

When the procedure was applied to the total crude dinitrophenylhydrazone mixture obtained from benzylpenicillin-free crystalline material, only a small amount of volatile aldehyde was recovered as the dinitrophenylhydrazone after the second oxidation step. Lack of material prevented complete purification of the derivative, but its analytical properties left little doubt that the constituting aldehyde was propionaldehyde and not acetaldehyde, and hence the penicillin from which this fragment was derived was 2-pentenylpenicillin. This result renders it all the more probable that the 1944 flavacidin was *n*-amylpenicillin and not 3-pentenylpenicillin.

An incidental result was the isolation of 2-furoic acid from a chromatographic side fraction. It is interesting to note that this acid was also encountered by McKee and MacPhillamy (13) in their original work on the factor later called flavacidin. It has furthermore been found in impure penicillin from *Penicillium notatum* (14). Since 2-furoic acid does not seem to be a common metabolic product of molds, it may have its origin in the corn steep liquor used as a constituent of the medium in all these instances.

EXPERIMENTAL

Microbiological Assays—Unless stated otherwise, the potency and unitage figures given in the following were obtained by plate assay with *Staphylococcus aureus* (Heatley) as the test organism and crystalline sodium benzylpenicillinate (Food and Drug Administration standard, 1667 units per mg.) as the standard. Differential assay denotes the ratio of the activity against *Bacillus subtilis* (rough strain) over that against *Staphylococcus aureus* as measured by plate assay (15). For some of the highly purified and crystalline preparations, the ratios of the minimal inhibiting concentrations for *Staphylococcus aureus* to *Bacillus brevis* to Organism E (16), as measured by the broth dilution method of Donovick, Lapedes, and Pansy (17), are given. This ratio, designated St:B:E in the following, is 1:1.9:4.2 for benzylpenicillin, 1:3.8:6.9 for 2-pentenylpenicillin, and (on the basis of the results obtained in this work with about 75 per cent pure potassium *n*-amylpenicillinate) in the neighborhood of 1:3.5:12 for *n*-amylpenicillin.

Fermentation and Extraction—The fermentation of *Aspergillus flavus* was carried out in a large fermentation tank, with a medium which contained besides the usual inorganic constituents (KH_2PO_4 1.0 per cent, magnesium sulfate 0.55 per cent, chalk 1.0 per cent) 1.5 per cent of cheese whey as the lactose source, and 6.2 per cent of corn steep liquor. The broth was harvested 73 hours after inoculation, when its pH was 7.3 and its potency 70 units per cc. It was worked up by the usual multiple extraction procedure (amyl acetate → buffer → chloroform → buffer → ether). The final ether solution, which contained 74 per cent of the activity in the filtered broth, was fractionally extracted with sodium bicarbonate solution in such a manner that the final pH of the first extract was 6.0 (Fraction I); that of the second, 6.5 (Fraction II); and that of the third, 7.2 (Fraction III). The sodium salt obtained from the first extract represented 62 per cent of the units present in the ether and had a potency of 720 units per mg.; the differential assay ratio was 0.9, indicating the presence of a considerable amount of benzylpenicillin (differential assay = 1.0). For the reason pointed out above, only this fraction was thoroughly examined chemically.[4]

Chromatographic Purification—A typical experiment is described. 25 gm. portions of the crude sodium salt from the extract at pH 6.0 were dissolved in 150 cc. of acetone containing 5 per cent of water, and chromatographed on a column (6.5 × 47 cm.) of sulfuric acid-washed alumina (pH 4.5 in water suspension). The chromatogram was developed by washing with 3 liters of the same solvent. The distribution of pigments and of activity in a typical chromatogram was as follows: brown top zone (A), 7 cm.; light tan zone (B), 16 cm.; orange band (C), 1 cm.; colorless zone (D), 5 cm.; yellow band (E), 1 cm.; pale yellow zone (F), 17 cm. The column was cut into three sections, each of which was eluted with 0.2 M phosphate buffer of pH 7.0 at 5°. The eluted materials were converted into sodium salts in the usual way. The top section, SI, comprising Zone A, contained 17 per cent of the activity; the middle section, SII (Zones B, C, and D), 45 per cent; the bottom section, SIII (Zones E and F), 24 per cent.

---

[4] The corresponding data for the other two fractions are as follows: Fraction II, 23.5 per cent of activity, 745 units per mg., differential assay, 0.80; Fraction III, 10 per cent of activity, 366 units per mg., differential assay, 0.70. Preliminary chromatographic and crystallization experiments were carried out on both fractions by Dr. C. Glaser of the Squibb Manufacturing Laboratories. The crystalline product obtained from Fraction II in poor yield resembled similar material from Fraction I, particularly in regard to its high content of benzylpenicillin. Fraction III yielded small amounts of crystalline products containing relatively little benzylpenicillin; the analytical, Craig, and microbiological data indicated that they consisted of mixtures of penicillins of the F type with some benzylpenicillin and probably also *n*-heptylpenicillin.

Sodium salts obtained from Section SII, which represented the purest material and contained the bulk of the activity, readily yielded crystals on treatment with dry acetone. However, when their high content of benzylpenicillin became apparent, crystallization at this stage was omitted; instead, the buffer eluate from Section SII was chilled, acidified to pH 2 with phosphoric acid, and extracted with ether. This ether solution was then used directly for the fractionation with triethylamine.

Isolation of Benzylpenicillin—To a dry ether solution (160 cc.) of free penicillin (1.6 gm., representing Section SII from the chromatographic fractionation of 5 gm. of Fraction I) there was added in small portions a 10 per cent ethereal solution of triethylamine. The oily deposits initially formed were discarded. After the addition of an excess of the reagent the solution was allowed to stand at 4° for 12 hours. The crystalline triethylamine salt was collected (522 mg.) and recrystallized from hot chlorobenzene (18). It melted at 131–134° (decomposition) and showed the following microbiological characteristics: potency, 1310 units per mg.; differential assay ratio, 0.93; St:B:E, 1:1.7:5.2. The analysis of the desiccator-dry preparation was low in carbon, indicating contamination with an F type of penicillin. A larger amount of crude triethylamine salt (SII-A, 15.8 gm., from 60 gm. of Fraction I) was therefore converted into the sodium salt by transfer through ether. A portion of the lyophilized product (5.5 gm.) was treated with dry acetone, and the resulting semicrystalline mass (1.25 gm.) was recrystallized from aqueous *n*-butanol in the usual manner (19). Further recrystallization from aqueous acetone gave 0.9 gm. of pure sodium benzylpenicillinate assaying 1640 units per mg. (average of thirteen assays) and giving a St:B ratio (minimal inhibiting concentrations) of 1:1.55.

$C_{16}H_{17}O_4N_2SNa$. Calculated, C 53.92, H 4.81; found, C 54.15, H 4.99

Isolation and Properties of Crystalline Salts from Benzylpenicillin-Free Fraction—The ethereal filtrate from the triethylamine salt SII-A was chilled and extracted with an ice-cold solution of phosphoric acid till all the triethylamine was removed. The ether phase was washed with water and then extracted with several portions of dilute aqueous ammonium hydroxide till the pH of the combined extracts was 6.5. The lyophilized ammonium salt, which assayed 1470 units per mg. and represented 25 per cent of the ether-extractable solids in the eluate SII, was crystallized by dissolving it in a minimum volume of acetone containing 5 per cent of water and adding absolute acetone in excess. The microbiological characteristics of the crystalline preparation (SII-B1), which did not differ materially from that of the amorphous product, were as follows: potency, 1510 units per mg. (sixteen assays); differential assay 0.63; St:B:E, 1:3.8:13. The analytical sample was dried at 37° *in vacuo* over phosphorus pentoxide (weight loss, 2.63 per cent).

$C_{14}H_{19}O_4N_2S\cdot NH_4$.	Calculated.	C 51.04, H 7.04
$C_{14}H_{21}O_4N_2S\cdot NH_4$.	"	" 50.69, " 7.60
	Found.	" 50.19, " 7.38
	"	" 50.13, " 7.49

The mother liquor yielded additional crystalline material of similar microbiological properties. The total amount (3.09 gm.) corresponded to 5.2 per cent of the weight of the starting material (Fraction I), or 11 per cent of its activity.

A 200 mg. sample, dissolved in 5 cc. of water, was hydrogenated at 30° in the presence of platinum oxide (500 mg.). After 3 hours 18.7 per cent of the amount of hydrogen which would have been required by pure ammonium pentenylpenicillinate had been consumed. Continued hydrogenation with fresh catalyst resulted in a small additional uptake, which raised the above figure to 21.8 per cent.

A portion of the ammonium salt was converted to the potassium salt by transfer through ether. Crystallization of the lyophilized material from aqueous acetone in the manner described above yielded a hygroscopic, crystalline product (SII-B2) assaying 1420 units per mg. and showing a St:B:E ratio of 1:3.0:11.2. The analytical sample lost 3.4 per cent of moisture on drying at 100° *in vacuo* over phosphorus pentoxide.

$C_{14}H_{19}O_4N_2SK$.	Calculated.	C 47.98, H 5.45, N 7.99, K 11.16
$C_{14}H_{21}O_4N_2SK$.	"	" 47.70, " 6.00, " 7.95, " 11.04
	Found.	" 47.85, " 6.02, " 7.90, " 11.25
	"	" 47.69, " 5.97, " 7.69, " 11.02

The counter-current distribution data obtained with this material are given in Fig. 1.

It will be noted that the experimental (weight and activity) curves coincide fairly well with the theoretical curve between Tubes 14 and 19, but markedly deviate from it below Tube 13 and, to a lesser extent, above Tube 20. Since it must be assumed that Tubes 14 to 19 contained for the most part *n*-amylpenicillin, the total amount of penicillin calculated from the ordinate values of the theoretical curve, in relation to the total amount of penicillin actually recovered from the tubes, may be taken as a measure of the content of n-amylpenicillin. The figure thus obtained, 74 per cent, is in good agreement with the value (72 per cent) calculated from the yield of crude n-caproic acid isolated from the acid hydrolysate of the crystalline ammonium salt (see below).

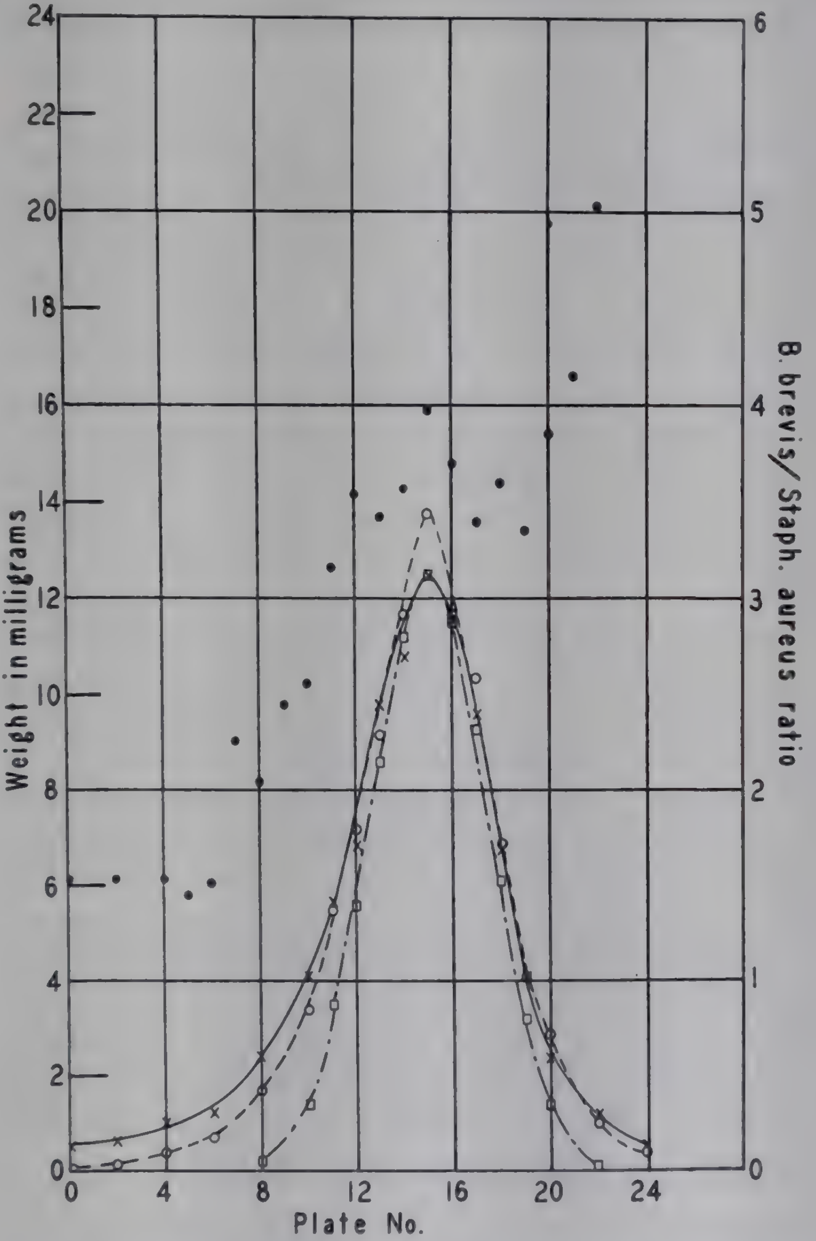


FIG. 1. Twenty-four tube counter-current distribution of potassium salt SII-B2. System, 2 M phosphate buffer (pH 4.8)-ether, 5°. ×, mg. of free penicillin, determined by weighing; ○, mg. of free penicillin, determined by assay (= (units)/1400); □, theoretical curve, calculated from the distribution coefficient, $K = 1.67$, and the weight of penicillin in Tube 15. ●, (*B. brevis*)/(*Staph. aureus*) ratio.

The deviation from the theoretical curve at Tubes 19 to 24 corresponds to the presence of 5.6 per cent of a component with a high distribution coefficient, possibly *n*-heptylpenicillin. The more slowly moving component revealed by the deviation at the left side of the maximum and accounting for the remaining 20 per cent was probably for the most part 2-pentenylpenicillin.

Spectrophotometric Procedure for Detection and Estimation of Benzylpenicillin in Mixtures—The absence of significant amounts (>5 per cent) of benzylpenicillin in the ammonium salt SII-B1 was proved by means of a spectrophotometric procedure, which utilizes the fact that the phenyl bands originating in the benzyl group become much better defined when this penicillin is hydrolyzed with alkali to benzylpenicilloic acid. Whereas in the ultraviolet absorption curve of benzylpenicillin the phenyl bands at 252, 258, and 264 mμ appear superimposed on the slope of the end-absorption, which is still very intense in that region, alkaline hydrolysis causes recession towards lower wave-lengths of the high band responsible for the end-absorption, so that the phenyl bands become well resolved, and their relative intensities approach more nearly those seen in the spectrum of phenylacetic acid. The extinction coefficient of the main phenyl band at 258.5 mμ can then serve as a rough measure of the benzylpenicillin content of a mixture, while in the curve of the unhydrolyzed preparation it cannot be so evaluated. A correction must be applied for the contribution at this wave-length of the residual end-absorption band; *i.e.*, the extinction at 258 mμ given by an alkali-hydrolyzed sample of a pure penicillin possessing an aliphatic R group (preferably *n*-heptylpenicillin, which can be most easily freed from benzylpenicillin). Reasonably good quantitative results were thus obtained on mixtures containing various proportions of sodium benzylpenicillinate and ammonium *n*-heptylpenicillinate, the purity of which had been checked by the Craig counter-current distribution. With such mixtures, the greatest deviation of the determined from the actual benzylpenicillin content was ±5 per cent of the total penicillin present, and so the smallest content which could be detected with certainty was about 5 per cent. Three illustrative absorption curves are given in Fig. 2.

The experimental procedure was as follows: An accurately weighed sample (about 10 mg.) of the penicillin salt to be examined was dissolved in 5.0 cc. of aqueous 0.2 N sodium hydroxide. After standing at room temperature for 30 minutes, the solution was neutralized with 5.0 cc. of 0.2 N hydrochloric acid, and used as such or after suitable dilution with 0.2 N sodium chloride solution for the determination of the ultraviolet characteristics in the region 245 to 270 mμ. When little or no benzylpenicillin was expected to be present, the extinctions were measured over that range in 0.5 mμ intervals and the complete curve was plotted; so that small deviations from the smooth, flat curve characteristic for benzylpenicillin-free preparations could be detected.

n-Caproic Acid from Ammonium Salt SII-B1—The ammonium salt (600 mg.) was hydrolyzed with boiling 20 per cent hydrochloric acid (25 cc.) for 18 hours, whereupon the solution was distilled with steam. The distillate was neutralized with sodium bicarbonate and extracted with ether. The neutral product recovered from the dried ether phase was a brown oil (43 mg.), most of which volatilized on standing for several days in the evacuated desiccator. This product was apparently a lactone formed under the influence of the hot mineral acid from an unsaturated acid, presumably the 3-hexenoic acid.

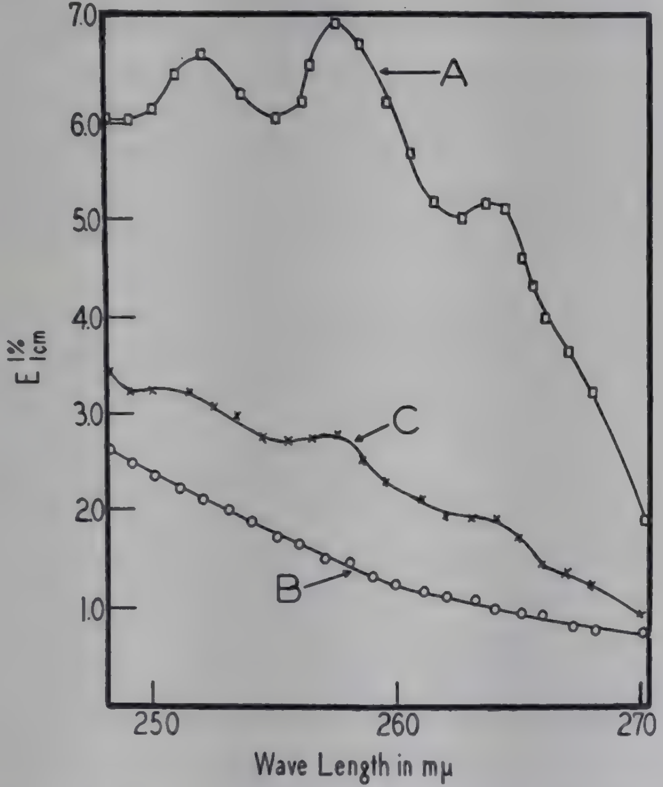


FIG. 2. Ultraviolet absorption curves of alkali-hydrolyzed penicillin. (1) Sodium benzylpenicillinate (Curve A); (2) ammonium *n*-heptylpenicillinate (Curve B); (3) mixture of (1) and (2), weight proportion 1:3 (Curve C).

The aqueous phase on acidification and extraction with ether yielded to the latter a liquid acid (152 mg.), which was purified by distillation *in vacuo* in a molecular still (b.p. 87° (bath temperature) at 9 mm.). When immersed in a freezing mixture, it crystallized and on thawing melted at −4° to −2°. The product did not react with alkaline permanganate or with bromine.

$C_6H_{12}O_4$. Calculated, C 62.04, H 10.41; found, C 62.02, H 10.31

The *p*-toluidide was prepared via the acid chloride, and after three recrystallizations from hexane melted at 74.5–75.5°. It did not depress the melting point of an authentic sample of *n*-caproic acid *p*-toluidide (75–76°).

$C_{13}H_{19}ON$. Calculated. C 76.08, H 9.33, N 6.83
Found. " 76.09, " 9.16, " 7.09

Penilloaldehyde 2,4-Dinitrophenylhydrazone from Potassium Salt SII-B2—A solution of the salt (95 mg.) in 0.1 N sulfuric acid (15 cc.) was boiled under reflux for 2 hours, and after cooling mixed with a 5 per cent mercuric chloride solution (10 cc.). The resulting precipitate (penicillamine mercury mercaptide) was filtered off and washed with water. The combined filtrate and washings after demercurization with hydrogen sulfide, filtration, and aeration with nitrogen gas yielded with excess dinitrophenylhydrazine reagent (in ethanol-4 N HCl, 1:1) 44 mg. of a precipitate which after two recrystallizations from 75 per cent ethanol melted at 182–182.5° (decomposition).

The material recovered from Tubes 14 to 20 of the counter-current distribution experiment was combined and treated in the same manner. The purified dinitrophenylhydrazone thus obtained melted at 182.5–183.5° (decomposition).

n-Caproylaminoacetaldehyde 2,4-Dinitrophenylhydrazone—The diethylacetal of the adehyde was prepared from *n*-caproyl chloride and aminoacetaldehyde diethyl acetal in pyridine-ether according to Bentley *et al.* (20). The compound was purified by fractional distillation (b.p. 155–160° at 10 mm.) and then treated with a saturated aqueous solution of 2,4-dinitrophenylhydrazone in 5 N hydrochloric acid at 0°. The melting point of the once recrystallized dinitrophenylhydrazone was 187.5–188° (decomposition) and remained constant on further recrystallization.

4-Hexenoylaminoacetaldehyde 2,4-Dinitrophenylhydrazone—4-Hexenoic acid was prepared from "hydrosorbic acid" by the procedure of Letch and Linstead (21). The constants of the methyl ester (b.p. 55–55.6° at 19 mm.; n_D^{20} 1.4248) and of the acid (b.p. 107–108° at 16 mm.; n_D^{20} 1.4389) were in agreement with those given by these authors. The *p*-toluidide (22), prepared via the acid chloride, melted at 104°.

$C_{13}H_{17}ON$. Calculated. C 76.96, H 8.57, N 6.81
Found. " 76.81, " 8.43, " 6.89

A mixture of 4-hexenoic acid (1.97 gm.) and thionyl chloride (2.6 gm.) was allowed to stand at room temperature for 24 hours. After removal of excess reagent by aeration with nitrogen the residual oil was cooled to 0° and mixed with an ice-cold solution of aminoacetaldehyde diethyl acetal (1.84 gm.) in absolute ether (20 cc.). The solution was allowed to stand at room temperature for 2 hours, whereupon it was filtered, washed with 20 per cent potassium carbonate solution and water, and dried over sodium sulfate. The residue obtained by removal of the solvent (4.0 gm.) was fractionally distilled *in vacuo*. The main fraction (b.p. 155–156° at 9 mm.) was treated with a saturated aqueous solution of 2,4-dinitrophenylhydrazine in 5 N hydrochloric acid at 0°. The resulting precipitate, after recrystallization from 60 per cent ethanol, melted at 185–186° (decomposition). The melting point did not change on further recrystallization.

$C_{14}H_{17}O_5N_5$.	Calculated.	C 50.15, H 5.11, N 20.9
	Found.	" 50.39, " 5.13, " 20.7

The melting points of the four dinitrophenylhydrazones prepared in this investigation, together with those of the penilloaldehydes from the 1944 flavacidin and from 2-pentenylpenicillin produced by *Penicillium notatum* (5), are listed in Table I. With the exception of IV, the preparations listed showed no melting point depression in mixture with one another. The melting points of the mixtures were generally those of the lower melting component, or slightly higher. Thus the mixtures of I and III and of I and V melted at 182–183°; that of I and VI, at 183–184°; that of III and V, at 181.5–183.5°; and that of the two synthetic compounds, V and VI, at 185–186°. In contrast, the penilloaldehyde dinitrophenylhydrazone from 2-pentenylpenicillin (IV) depressed the melting point of III to 174–175.5°, and that of the synthetic compound V to 172–172.5°.

TABLE I

2,4-Dinitrophenylhydrazone of	M.p.
	°C.
Penilloaldehyde from K salt SII-B2 (I)	182 –182.5
" " " " Tubes 14-20 (II)	182.5–183.5
" " 1944 flavacidin (III)	181.5–182*
" " 2-pentenylpenicillin (IV)	178 –180
n-Caproylaminoacetaldehyde (V)	187.5–188
4-Hexenoylaminoacetaldehyde (VI)	185 –186

* Originally given as 180–180.5° (3).

Degradation of 4-Hexenoic Acid and Derivatives to Acetaldehyde. (*a*) *With 4-Hexenoic Acid*—To a solution of 4-hexenoic acid (445 mg.) in absolute ether (12 cc.) there was added osmium tetroxide (1.0 gm.) in 5 cc. of the same solvent. A brown precipitate formed immediately. The mixture was allowed to stand at room temperature for 2 days in the dark. The residue obtained by removal of the solvent *in vacuo* was decomposed by boiling with a solution of sodium sulfite (15 gm.) in 200 cc. of 50 per cent aqueous ethanol for 4 hours. The resulting precipitate was removed by filtration and leached with four 50 cc. portions of ethanol. The residue obtained by evaporation of the combined filtrate and alcoholic solutions was freed from inorganic salts by repeated extraction with absolute ethanol. The extracts were brought to dryness *in vacuo*, yielding a yellow oil which was used without further purification for the reaction with periodic acid.

The consumption of periodate was determined by the method of Rappaport, Reifer, and Weinmann (23); 17.7 mg. consumed in fast reaction 26.6 mg. of KIO_4 (24 hour value); required for 4,5-dihydroxyhexanoic acid, 27.7 mg.

The glycol (220 mg.) was treated with periodic acid (620 mg.) in aqueous solution (12.5 cc.). The volatile reaction products were entrained in a current of nitrogen which passed through a 0.4 per cent solution of 2,4-dinitrophenylhydrazine in ethanol-4 N hydrochloric acid, 1:1, till no further precipitate was formed. The crude hydrazone (collected after 24 hours aeration, 244 mg., 73.7 per cent of the calculated amount) melted at 136° (decomposition) after drying at room temperature. On recrystallization from aqueous ethanol, it yielded three successive crops of crystals differing in their melting points (147–151°; 142–143°; 145–149°). These were combined and chromatographed in chloroform solution on alumina. The bulk of the material was recovered from the bottom of the column by elution with ethanol as two fractions melting at 138–140° and 141–142°, respectively. Rechromatographing the combined fractions in benzene-hexane solution indicated that it was essentially homogeneous, though it now melted at 145–150°. It appears that this preparation consisted preponderantly of the low melting modification of acetaldehyde 2,4-dinitrophenylhydrazone (24), for on drying at 100° *in vacuo* the melting point rose steadily till it became constant at 154–156°.

$C_8H_8O_4N_4$. Calculated. C 42.86, H 3.60, N 25.0
Found. " 42.68, " 3.51, " 24.8

(*b*) *With 4-Hexenoic Acid p-Toluidide*—A solution of the toluidide (1.444 gm.) in absolute ether (120 cc.) containing osmium tetroxide (2 gm.) was allowed to stand in the dark for 4 days, whereupon it was worked up as described under (*a*), but with double the amounts of sodium sulfite and solvents. The final product was a brown syrup which was decolorized with charcoal in ethanol and then crystallized from hot water. The *4,5-dihydroxycaproic acid p-toluidide* thus obtained melted at 150–150.5° (decomposition). Recrystallization did not raise the melting point. The yield of pure product was 780 mg. (46 per cent).

$C_{13}H_{19}O_3N$. Calculated. C 65.80, H 8.07, N 5.94
Found. " 65.77, " 8.31, " 5.77

The toluidide (221 mg.) was dissolved in ethanol (2 cc.) and a solution of periodic acid (314 mg.) in water (4 cc.) was added. Aeration with nitrogen after 5 hours standing into dinitrophenylhydrazine reagent yielded 114 mg. of the acetaldehyde derivative, m.p. 147–150°, and an additional 28 mg. after 24 hours, the total yield corresponding to 65 per cent of the calculated amount.

(*c*) *With 4-Hexenoylaminoacetaldehyde 2,4-Dinitrophenylhydrazone*—To a solution of the hydrazone (160 mg.) in 7 cc. of dry pyridine (25) osmium tetroxide (250 mg.) dissolved in absolute ether (4 cc.) was added. After 2 days the mixture was worked up as described under (*a*). The crude reaction product (120 mg.) was used without further purification for the reaction with periodic acid (151 mg.). The solvent used was 23.5 per cent aqueous ethanol (8.5 cc.). After 6.5 hours 31 mg. and after 24 hours an additional 20 mg. of acetaldehyde dinitrophenylhydrazone, m.p. 143–144°, were collected. Recrystallization from aqueous ethanol (m.p. 149–150°) followed by heating at 100° *in vacuo* raised the melting point to 153–157°. The identity of the product was confirmed by elementary analysis.

A blank experiment carried out with the above amounts of periodic acid and 23.5 per cent ethanol yielded 17.5 mg. of a brown precipitate which possibly contained some acetaldehyde dinitrophenylhydrazone derived from the ethanol by an abnormal type of oxidation. In a similar blank run with aqueous dioxane no such precipitate was formed. This solvent was therefore used in the periodic acid step of the degradation experiment described below.

Oxidative Degradation of Penilloaldehyde Dinitrophenylhydrazone Mixture from Benzylpenicillin-Free Fraction—The starting product was a crystalline, benzylpenicillin-free ammonium salt which was prepared from Fraction I in the same manner as the ammonium salt SII-B1. It was degraded to the penilloaldehyde as described for the potassium salt SII-B2, except that the demercurization with hydrogen sulfide prior to the addition of the dinitrophenylhydrazine reagent was omitted. The crude dinitrophenylhydrazone (1.3 gm., m.p. 170–173°, decomposition) was treated with osmium tetroxide in pyridine-ether as in the procedure described in section (*c*). The reaction product, isolated in the usual manner, was a yellow oil weighing 750 mg. It was dissolved in pure dioxane (20 cc.), and after the addition of an aqueous solution (40 cc.) of periodic acid (1.4 gm.) a stream of nitrogen was passed through the clear solution into a receiver containing a 0.4 per cent solution of dinitrophenylhydrazine in ethanol-2 N hydrochloric acid, 1:1. The orange-colored crystals collected after 24 hours of aeration weighed 16 mg. and melted at 133–134° (decomposition, hot stage). They were recrystallized first from absolute ethanol and then from glacial acetic acid (m.p. 142–144°, hot stage). Lack of material prevented further purification. A mixture with an authentic specimen of propionaldehyde 2,4-dinitrophenylhydrazone (m.p. 148–151°) melted at 143–145° (decomposition, hot stage).

$C_9H_{10}O_4N_4$.	Calculated.	C 45.38,	H 4.23,	N 23.5
$C_8H_8O_4N_4$.	"	" 42.86,	" 3.60,	" 25.0
	Found.	" 44.40,	" 3.92,	" 23.6

On a molar basis, the yield of propionaldehyde from the dinitrophenylhydrazone mixture was 1.85 per cent, which corresponds to only a small fraction of the assumed pentenylpenicillin content of the comparable ammonium salt SII-B1 (about 20 per cent). This discrepancy is undoubtedly in part due to losses sustained in the three steps of the degradation procedure; the yield of penilloaldehyde dinitrophenylhydrazone was only 60 per cent of the amount calculated for ammonium *n*-amylpenicillin, and, to judge from the experiment with 4-hexenoylaminoacetaldehyde dinitrophenylhydrazone, in which the yield of the acetaldehyde derivative was 47.5 per cent, even greater losses probably occurred in the following two oxidative steps. Moreover, some fractionation, entailing a comparatively greater loss of the unsaturated component, may have taken place in the preparation of the penilloaldehyde hydrazone mixture. However, in view of the complex procedure employed in the preparation of the benzylpenicillin-free salt from Fraction I, it is also quite possible that the pentenylpenicillin content of the ammonium salt used for the above experiment was markedly lower than that of SII-B1, and that this was the chief reason for the unexpectedly low over-all yield of propionaldehyde.

Isolation of 2-Furoic Acid—In a chromatographic experiment starting from 30 gm. of Fraction I, the dark brown band forming the lower part of Zone A was eluted separately with phosphate buffer. The eluate was subjected to the same procedure, inclusive of the precipitation with triethylamine, as the middle section of the chromatogram (Section SII). The lyophilized ammonium salt (1.2 gm., 435 units per mg.) obtained from the triethylamine filtrate on treatment with absolute acetone yielded crystalline material which was recrystallized from aqueous acetone in the usual manner. The resulting crystalline salt (408 mg., m.p. 188–189°, decomposition) was biologically inactive, and was identified as ammonium 2-furoate by absorption spectrum ($\epsilon_{max.}$ 11,000 at 245 mμ, in water), analysis, and conversion to the free acid.

$C_5H_7O_3N$.	Calculated.	C 46.51,	H 5.46,	N 10.85
	Found.	" 46.72,	" 5.35,	" 10.54

The acid melted at 128–129° and did not depress the melting point of an authentic sample of 2-furoic acid.

$C_5H_4O_3$. Calculated, C 53.58, H 3.60; found, C 53.89, H 3.81

DISCUSSION

The results of this investigation show that *Aspergillus flavus* under suitable culture conditions equals *Penicillium notatum* in its capacity to elaborate various penicillin species simultaneously. Two of these, benzylpenicillin and *n*-amylpenicillin, were conclusively identified, and satisfactory evidence for the presence of a third, 2-pentenylpenicillin, was adduced. It is noteworthy that *n*-amylpenicillin was first encountered in nature as the metabolic product of another *Aspergillus* species, *Aspergillus giganteus*. The partially purified penicillin-like antibiotic obtained from this source by Philpot (11) in 1943 was originally termed gigantic acid; it was subsequently shown to be *n*-amylpenicillin by conversion to the penillic acid and crystallographic identification of the latter with "dihydro-I-penillic acid" (10). Much more recently Salivar, Bogert, and Brown (12) have demonstrated the occurrence of *n*-amylpenicillin in submerged culture filtrates of *Penicillium notatum* (strain Q-176) by isolation and degradation to *n*-caproic acid.

Though it is difficult to estimate the proportion in which the three identified penicillins were present, there is no doubt that benzylpenicillin preponderated, not only in the crude sodium salt fraction examined but also in the total mixture extracted from the broth. In contrast, the crystalline flavacidin sodium salt isolated in 1944 contained only small amounts of benzylpenicillin, although the chromatographic and crystallization procedures used had proved to be highly effective in the earlier isolation of this entity from *Penicillium notatum* broth. The greater abundance of this species in the present batch is probably to be ascribed to the use of lactose instead of brown sugar in the culture medium, a change which may have resulted in better utilization of the benzylpenicillin precursors supplied with the corn steep liquor. *Aspergillus flavus* is not the only representative of the genus *Aspergillus* capable of producing this penicillin. Arnstein and Cook (26) have recently identified "parasiticin," the antibiotic produced by *Aspergillus parasiticus* in surface culture on a medium containing glucose and corn steep liquor, as benzylpenicillin.

Salivar, Bogert, and Brown (12) in their detailed study of crystalline ammonium salt mixtures from *Penicillium notatum* (strain Q-176) demonstrated the presence in their material of a penicillin closely allied with 2-pentenylpenicillin, which on the basis of our original work on flavacidin they assumed to be 3-pentenylpenicillin. This product showed a somewhat higher distribution coefficient in the Craig ether-buffer system than did 2-pentenylpenicillin, but, like the latter, yielded on acid hydrolysis the lactone of 4-hydroxycaproic acid. Since the lactone could arise from either 3- or 4-hexenoic acid, it appeared possible that 3-pentenylpenicillin was involved. However, since the fraction in question may have contained some 2-pentenylpenicillin, and the yield of lactone relative to that obtained from the fraction definitely identified as 2-pentenylpenicillin was not stated, the evidence on this point is far from conclusive. Nevertheless, it is quite possible that future work may yet definitely establish the occurrence of 3-pentenylpenicillin in the penicillin mixtures produced by *Penicillium notatum*, or for that matter in such derived from *Aspergillus flavus*. In any case, the distinctive term "flavacidin," which was retained merely for convenience of reference pending conclusive identification of the entity in question, should now be abandoned.

We are indebted to Mr. R. Van Winkle, general superintendent of manufacture, and Dr. C. Glaser of the Antibiotics Manufacturing Division, for making available to us the starting material and the pertinent production and microbiological data; to Dr. R. Donovick of the Division of Microbiology, The Squibb Institute for Medical Research, for the microbiological assays on the purified and crystalline products; to Dr. Nettie Coy of the Division of Development, E. R. Squibb and Sons, for the ultraviolet absorption measurements; and to Mr. J. F. Alicino of this Institute for the microanalyses.

SUMMARY

Aspergillus flavus grown in submerged culture on a medium containing lactose and supplemented with corn steep liquor produced a mixture of penicillins, in which benzylpenicillin greatly preponderated. The crystalline fractions obtained after removal of the benzylpenicillin were shown to consist for the most part of *n*-amylpenicillin. Evidence has been adduced by oxidative degradation for the presence in these fractions of small amounts of 2-pentenylpenicillin.

The 2,4-dinitrophenylhydrazones of *n*-caproylaminoacetaldehyde and of 4-hexenoylaminoacetaldehyde do not mutually depress their melting points, and give practically identical x-ray diffraction patterns. In view of this finding it appears probable that the "flavacidin" which was isolated in 1944 (1, 3) was *n*-amylpenicillin and not, as was then assumed, 3-pentenylpenicillin.

BIBLIOGRAPHY

1. Fried, J., Koerber, W. L., and Wintersteiner, O., *J. Biol. Chem.*, **163**, 341 (1946).
2. Clarke, H. T., Johnson, J. R., and Robinson, R., The chemistry of penicillin, Princeton, in press.
3. Squibb Institute for Medical Research, S *14* (*cf.* (2) p. 104).
4. Abraham, E. P., Baker, W., Chain, E., and Robinson, R., PEN *97* ((2) p. 12).
5. Squibb Institute for Medical Research, S *5* ((2) p. 105).
6. MacPhillamy, H. B., Wintersteiner, O., and Alicino, J. F., S *3* ((2) p. 83).
7. Heyden Chemical Corporation, H *2* ((2) p. 90).

8. Craig, L. C., Hogeboom, G. H., Carpenter, F. H., and du Vigneaud, V., *J. Biol. Chem.*, **168**, 665 (1947).
9. Williamson, B., and Craig, L. C., *J. Biol. Chem.*, **168**, 687 (1947).
10. Catch, J. R., Cook, A. H., Elvidge, J. A., Hall, R. H., and Heilbron, I. M., PEN **99**, Sept. 29 (1943).
11. Philpot, F. J., *Nature*, **152**, 725 (1943).
12. Salivar, C. J., Bogert, V. V., and Brown, E. V., Report presented at the Conference on Antibiotic Research, Washington, D. C., Jan. 31 (1947), under the auspices of the Antibiotics Study Section, National Institute of Health.
13. McKee, C., and MacPhillamy, H. B., *Proc. Soc. Exp. Biol. and Med.*, **53**, 247 (1943).
14. Merck and Company, Inc., Report for Nov., 1942–Feb., 1943 (*cf.* (2) p. 53).
15. Schmidt, W. H., Ward, G. E., and Coghill, R. D., *J. Bact.*, **49**, 411 (1945).
16. Higuchi, K., and Peterson, W. H., *Ind. and Eng. Chem., Anal. Ed.*, **19**, 68 (1947).
17. Donovick, R., Lapedes, D., and Pansy, F., *J. Bact.*, **54**, 425 (1947).
18. Chas. Pfizer and Company, P *24*, Apr. 9 (1945).
19. Dutcher, J. D., MacPhillamy, H. B., Stavely, H. E., and Wintersteiner, O., S *3a* ((2) p. 88).
20. Bentley, R., Catch, J. R., Cook, A. H., Elvidge, J. A., Hall, R. H., and Heilbron, I. M., PEN *110* ((2) p. 49).
21. Letch, R. A., and Linstead, R. P., *J. Chem. Soc.*, 1994 (1934).
22. Fichter, F., and Pfister, A., *Ber. chem. Ges.*, **37**, 1997 (1904).
23. Rappaport, F., Reifer, J., and Weinmann, H., *Mikrochim. acta*, **1**, 290 (1937).
24. Allen, C. F., *J. Am. Chem. Soc.*, **52**, 2955 (1930).
25. Criegee, R., Marchand, B., and Wannowius, H., *Ann. Chem.*, **550**, 99 (1942).
26. Arnstein, H. R. V., and Cook, A. H., *Brit. J. Exp. Path.*, **28**, 94 (1947).

BENZYLPENICILLENIC ACID AS AN INTERMEDIATE IN THE SYNTHESIS OF BENZYLPENICILLIN (PENICILLIN G)*

By FREDERICK H. CARPENTER, ROBERT A. TURNER, AND VINCENT DU VIGNEAUD

(*From the Department of Biochemistry, Cornell University Medical College, New York City*)

(Received for publication, July 23, 1948)

During the war several English and American laboratories participated in a study of the structure and synthesis of penicillin (1, 2). In this work it was noted that when the methyl ester of benzylpenicillin (I) (see Fig. 1) was treated with mercuric chloride in ether solution and the resulting mercury derivative (II) was decomposed with hydrogen sulfide, a neutral, amorphous product was obtained. This crude product possessed an absorption peak in the ultraviolet at 320 mμ (E_M = 13,700)[1] and was degraded by sodium hydroxide to the sodium salt of 2-benzyl-4-hydroxymethylene-5(4)-oxazolone. For these and other reasons the product was assigned structure (III) and was given the trivial name methyl D-benzylpenicillenate (3).

After the close of the war, studies were continued in this Laboratory on the synthesis and the mechanism of synthesis of benzylpenicillin from D-penicillamine hydrochloride (V) and 2-benzyl-4-methoxymethylene-5(4)-oxazolone (IV) (4, 5). It was found that when the two compounds were allowed to react in pyridine containing triethylamine, a biologically inactive, amorphous product was obtained. However, when this product was heated in pyridine containing pyridinium chloride, benzylpenicillin was formed in small yield. The intermediate product possessed an ultraviolet absorption spectrum similar to that described for natural methyl D-benzylpenicillenate (III).[2] So far attempts to isolate the intermediate compound in crystalline form have not been successful. During the course of fractionation studies on this product, the formation of D-benzylpenillic acid (VI) was encountered (6). This D-benzylpenillic acid was identical with that formed by rearrangement of benzylpenicillin.

---

* This work was supported in part by a research grant from the National Institute of Health.

[1] Throughout this paper E_M is the molar absorption coefficient and is equal to D/cd, where D is $\log I_0/I$, c is concentration in moles per liter, and d is cell thickness in cm.

[2] In order to simplify phraseology in this article, D-benzylpenicillenic acid which has been prepared by rearrangement of benzylpenicillin is called "natural" D-benzylpenicillenic acid in contrast to that which has been prepared by total synthesis.

When DL-penicillamine hydrochloride was used instead of D-penicillamine hydrochloride in the condensation with the oxazolone (IV) in pyridine containing triethylamine, it was possible to isolate a crystalline compound from the reaction mixture. Since this compound had an absorption peak at 322.5 mμ (E_M = 26,000 to 28,000) and other properties in agreement with those of natural methyl D-benzylpenicillenate, it was called DL-benzylpenicillenic acid (IIIa) (7).

The crystalline DL-benzylpenicillenic acid (IIIa) rearranged in alcoholic solution to give a racemic benzylpenillic acid (VI). When the DL-benzylpenicillenic acid was heated in pyridine containing pyridinium chloride, antibiotic activity was produced, and furthermore in an amount proportional to the amount of D-benzylpenicillenic acid present. This ability to produce antibiotic activity was retained unchanged through repeated recrystallizations of the DL-benzylpenicillenic acid (7).

Since these results indicated that benzylpenicillenic acid (IIIa) was an intermediate in the synthesis of penicillin, it was desirable to demonstrate that the synthetic D-benzylpenicillenic acid was identical with natural D-benzylpenicillenic acid formed by rearrangement of benzylpenicillin.

Previous attempts in this and other Laboratories to obtain either natural or synthetic D-benzylpenicillenic acid in crystalline form had been unsuccessful (3, 5). Thus we were faced with the problem of trying to establish identity between two amorphous compounds. Since DL-benzylpenicillenic acid had been obtained in crystalline form, it occurred to us that amorphous D-benzylpenicillenic acid, either natural or synthetic, might be converted to a crystalline product by mixing it with an equivalent amount of amorphous L-benzylpenicillenic acid. Thus one might expect to obtain, on the one hand, a crystalline DL-benzylpenicillenic acid in which the D moiety arose by rearrangement of benzylpenicillin and, on the other hand, a crystalline DL-benzylpenicillenic acid in which the D moiety arose by synthesis from D-penicillamine (V) and the oxazolone (IV). In both cases, the L moiety would consist of synthetic L-benzylpenicillenic acid. If these two crystalline DL-benzylpenicillenic acids could be shown to be identical, then it would necessarily follow that the natural D-benzylpenicillenic acid was identical with the synthetic D-benzylpenicillenic acid.

Methyl benzylpenicillenate (III) had been prepared by the action of mercuric chloride on the methyl ester of benzylpenicillin (3). Since saponification of a compound as unstable as methyl benzylpenicillenate did not seem feasible, it was decided to investigate the action of mercuric chloride on sodium benzylpenicillin. If the reaction with sodium benzylpenicillin proceeded in a fashion analogous to that with the ester, one would expect to obtain D-benzylpenicillenic acid directly. Investigators at the Abbott Laboratories (3) had reported that treatment of sodium benzylpenicillin in aqueous solution with mercuric chloride resulted in the precipitation of a mercury derivative. This product possessed an absorption peak in dioxane at 320 mμ (E_M = 15,400) (3). The material was probably the mercury derivative (IIa) of D-benzylpenicillenic acid, and therefore a study of this reaction was initiated. Conditions were developed by which a mercury derivative (IIa) with an absorption peak in dioxane at 320 mμ (E_M = 18,000 to 19,000) and in ethanol at 342 mμ (E_M = 21,000 to 23,000) could be obtained from benzylpenicillin. This mercury derivative (IIa) was successfully converted to crude D-benzylpenicillenic acid (IIIa) by treating a suspension of the compound in a water-ethyl acetate mixture with hydrogen sulfide. The amorphous D-benzylpenicillenic acid which was isolated from the ethyl acetate layer possessed an absorption peak at 322.5 mμ (E_M = 17,600). When this crude D-benzylpenicillenic acid was allowed to stand in methanol, it rearranged to D-benzylpenillic acid (VI). In this respect it behaved similarly to the D-benzylpenicillenic acid prepared by synthesis from D-penicillamine (V) and the oxazolone (IV) (6).

Preliminary experiments were undertaken to determine whether crystalline DL-benzylpenicillenic acid could be obtained from a mixture of amorphous, synthetic D- and L-benzylpenicillenic acids. At first some difficulty was encountered in obtaining crystalline material upon admixture of the D and L compounds in solution. Presumably this was due to impurities present in these preparations. This difficulty was eliminated to a large degree when a method of partial purification of the crude penicillenic acids, based upon solvent extraction, was devised. It was noted that when a chloroform solution of the crude benzylpenicillenic acid was shaken with an equal volume of 2 M phosphate buffer solution at pH 5.4, a portion of the impurity went into the buffer layer. Although some of the benzylpenicillenic acid was either extracted or destroyed by this procedure, the benzylpenicillenic acid remaining in the chloroform layer was considerably purer than the starting material. The use of this information made it possible to isolate crystalline DL-benzylpenicillenic acid from a mixture of the synthetic D and L compounds.

Natural D-benzylpenicillenic acid was mixed with an equivalent amount of synthetic L-benzylpenicillenic acid. After a chloroform solution of this mixture had been purified by the extraction procedure, DL-benzylpenicillenic acid was isolated from the solution in crystalline form. This DL-benzylpenicillenic acid was identical in melting point, mixed melting point, and in infra-red and ultraviolet absorption spectra with the DL-benzylpenicillenic acid synthesized from DL-penicillamine (7) and also with material prepared from a mixture of synthetic D- and L-benzylpenicillenic acids. In addition, the DL-benzylpenicillenic acid (IIIa), in which the D moiety was natural, rearranged in methanolic solution to racemic benzylpenillic acid (VI).

When the DL-benzylpenicillenic acid containing natural D-benzylpenicillenic acid was heated in pyridine and pyridinium chloride, antibiotic activity was produced. The amount of antibiotic activity was equal, within experimental error, to that produced under similar conditions from synthetic DL-benzylpenicillenic acid. Synthetic L-benzylpenicillenic acid did not give rise to antibiotic activity under these conditions. Therefore, in the case of the DL-benzylpenicillenic acid, the activity must have arisen entirely from the D moiety of the compound.

These results prove that synthetic D-benzylpenicillenic acid is identical in all respects with natural D-benzylpenicillenic acid. Moreover the production of antibiotic activity, previously shown (2) to be due to benzylpenicillin, in identical amounts from two samples of crystalline DL-benzylpenicillenic acid in which the D moiety was prepared in two altogether different ways indicates beyond a reasonable doubt that *benzylpenicillenic acid, and not a small impurity present in the preparation, is an intermediate in the synthesis of benzylpenicillin* from penicillamine (V) and the oxazolone (IV). In any event, these data, in connection with those of other experiments cited above (2, 4, 5), demonstrate that benzylpenicillin may be rearranged to an antibiotically inactive product which under certain conditions can be converted in small part back to benzylpenicillin. The relationships discussed here are illustrated on the basis of the β-lactam formula for penicillin in Fig. 1.

The present communication also contains evidence as to the nature of the racemic benzylpenillic acid obtained by rearrangement of DL-benzylpenicillenic acid (7). Since there are three asymmetric carbon atoms in benzylpenillic acid, there are four possible racemic forms of this compound. Admixture of equal quantities of synthetic D- and L-benzylpenillic acids gave rise to crystalline DL-benzylpenillic acid that was identical with the material prepared by rearrangement of DL-benzylpenicillenic acid. These results demonstrate that the racemic benzylpenillic acid formed by rearrangement of DL-benzylpenicillenic acid contains a D moiety which is identical with the D-benzylpenillic acid produced by rearrangement of D-benzylpenicillin.

It should be pointed out that, since benzylpenicillenic acid (IIIa) can rearrange to benzylpenillic acid (VI), it is possible that the rearrangement of D-benzylpenicillin (Ia) to D-benzylpenillic acid (VI) in aqueous solution at pH 2 (8) takes place through the intermediate formation of D-benzylpenicillenic acid (IIIa).

EXPERIMENTAL[3]

D- *and* L-*Benzylpenicillenic Acids*—To a mixture of 3.04 gm. (0.015 mole) of L-penicillamine hydrochloride hydrate and 3.04 gm. (0.014 mole) of

---

[3] All melting points are corrected capillary melting points.

2-benzyl-4-methoxymethylene-5(4)-oxazolone were added 225 cc. of pyridine, and solution was effected by swirling the mixture. After addition of 24 cc. of triethylamine, the mixture was heated at 65–70° for 20 minutes.

The yellow solution was distilled *in vacuo* in a stream of nitrogen at a bath temperature of 50° until the solvents were removed. A solution of the residue in 300 cc. of chloroform was shaken with 150 cc. of 2 M phosphate buffer solution at pH 1.6 (prepared by admixture of equal volumes of 2 M H_3PO_4 and 2 M NaH_2PO_4). Then the chloroform layer was shaken for 1 minute with two 150 cc. portions of 2 M phosphate buffer solution at pH 5.4.[4] The separated chloroform layer was dried over anhydrous $MgSO_4$ for 20 minutes, filtered by suction, and distilled *in vacuo* almost to dryness. The residual gum was dissolved in 30 cc. of chloroform and added gradually

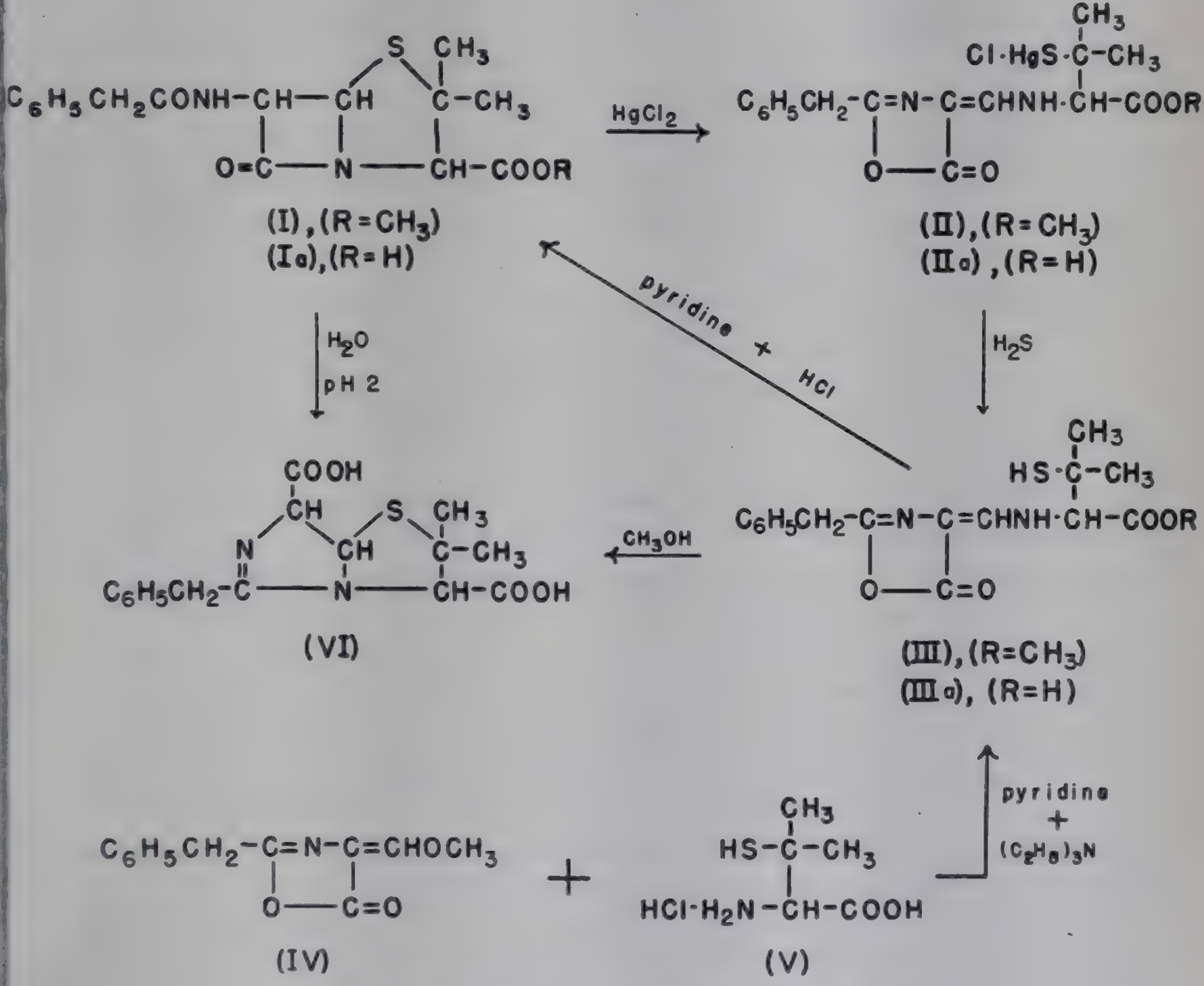


Fig. 1. Some reactions of benzylpenicillenic acid

to 600 cc. of agitated hexane. The precipitate which formed was immediately filtered and dried *in vacuo* at room temperature. The weight of amorphous L-benzylpenicillenic acid ranged from 3.10 to 3.65 gm. (66 to 78 per cent); E_M = 15,900 to 17,000 at 320 mμ in 95 per cent ethanol.[1] The maximum in the ultraviolet absorption peak occurred at 322.5 mμ. The specific rotation of the product varied slightly with different preparations, having an average value of about $[\alpha]_D^{22} = -82°$ (1.3 per cent solution in 95 per cent ethanol). Since the rotation of ethanolic solutions of either of the enantiomorphs of benzylpenicillenic acid gradually increased in value with time, the rotations were determined as soon as possible (within 15 minutes) after the solutions had been prepared.

---

[4] This buffer solution was prepared by admixture of 81 volumes of 2 M NaH_2PO_4 with 19 volumes of 2 M K_2HPO_4. The resulting solution gave a pH of 5.4 when measured without dilution with the glass electrode.

D-Benzylpenicillenic acid was prepared from D-penicillamine hydrochloride hydrate in the manner described for the L acid. The specific rotation was approximately equal in amount but opposite in sign to that found for L-benzylpenicillenic acid.

Purification of DL-*Benzylpenicillenic Acid by Extraction*—Synthetic D- and L-benzylpenicillenic acids having approximately the same absorption at 320 mμ (E_M = 16,500) were selected for this experiment. A mixture of 100 mg. of each compound was dissolved in 50 cc. of distilled chloroform. The chloroform solution was shaken for 1 minute with 50 cc. of 2 M phosphate buffer solution at pH 5.4.[4] Aliquots for determination of ultraviolet absorption were removed from the chloroform solution before and after the extraction. The results of the absorption measurements indicated that 21 per cent of the benzylpenicillenic acid was removed or destroyed by the extraction. After the chloroform layer had been dried over anhydrous $MgSO_4$, it was filtered and concentrated to dryness *in vacuo*. The residue weighed 98 mg. (49 per cent) and possessed a molar absorption at 320 mμ of E_M = 20,100. Thus the extraction removed from the chloroform 51 per cent of the material on a weight basis but only 21 per cent of the benzylpenicillenic acid on an absorption basis. An additional extraction with the pH 5.4 buffer solution did not effect appreciable further purification.

Preparation of DL-*Benzylpenicillenic Acid by Admixture of Synthetic Enantiomorphs*—To 300 cc. of ice-cold chloroform were added 0.73 gm. of amorphous L-benzylpenicillenic acid (E_M = 15,900 at 320 mμ, $[\alpha]_D^{22} = -82°$) and 0.70 gm. of amorphous D-benzylpenicillenic acid (E_M = 16,600 at 320 mμ; $[\alpha]_D^{22} = +80°$). These quantities were equivalent on the basis of molar absorption coefficients. The solution was shaken for 1 minute with 100 cc. of ice-cold 10 per cent H_3PO_4, and then was shaken with 300 cc. of ice-cold 2 M phosphate buffer solution at pH 5.4.[4] After 10 minutes (to allow complete separation of phases) the lower layer was drawn off, placed in an ice bath, and dried with anhydrous $MgSO_4$ for 20 minutes. The desiccant was then filtered by suction, and the clear, yellow solution was evaporated *in vacuo* in the absence of air of ebullition in a bath at 40–60°. Before half of the solvent was evaporated, the solution became cloudy and crystallization commenced. The mixture was concentrated to a volume of about 10 cc., allowed to stand in an ice bath for 40 minutes, and filtered. The white crystals were washed three times with a total of 5 cc. of distilled chloroform and dried *in vacuo* at room temperature; weight, 328 mg. (23 per cent); m.p. 131–133° (with decomposition); E_M = 24,600 at 320 mμ in 95 per cent ethanol.

When 298 mg. of this material were warmed for 5 minutes in 15 cc. of dry ethyl acetate and finally boiled for a minute, all but a trace of substance dissolved. The solution was filtered with the aid of gentle suction into a tared centrifuge tube and left in the cold. After 18 hours the white crystals were collected by centrifugation and dried *in vacuo;* weight, 178 mg. (60 per cent); m.p. 136–137° (with decomposition); E_M = 26,600 at 320 mμ in 95 per cent ethanol; absorption peak located at 322.5 mμ; $[\alpha]_D^{22} = 0°$ (0.35 per cent solution in 95 per cent ethanol). The melting point of this DL-benzylpenicillenic acid was not depressed upon admixture with DL-benzylpenicillenic acid prepared from DL-penicillamine (7).

TABLE I

Reaction of Mercuric Chloride with Sodium Benzylpenicillin

Concentration of reactants	Yield	E_M at 320 mμ
mole per l.	*per cent*	
0.0833	92	14,500
0.0417	89	16,000
0.0208	91	17,600
0.0104	85	18,500

Effect of Concentration on Reaction of Mercuric Chloride with Sodium Benzylpenicillin—Samples of sodium benzylpenicillin (0.25 mM) were dissolved in various amounts of water. An aqueous solution of mercuric chloride containing 0.25 mM was added to each penicillin solution and the final volume was noted. The solutions were allowed to stand at room temperature (25°) for 2 hours and then refrigerated at 5° for 16 hours. The precipitates of the mercury derivative (IIa) were collected by filtration and dried *in vacuo* over phosphoric anhydride at room temperature. The yield and molar absorption coefficient of each material at 320 mμ in dioxane are shown in Table I.

Preparation of Mercury Derivative (IIa) from Sodium Benzylpenicillin—To 1.78 gm. (0.005 mole) of sodium benzylpenicillin dissolved in 100 cc. of water were added 1.49 gm. (0.0055 mole) of mercuric chloride dissolved in 100 cc. of water. The clear solution gradually developed turbidity while standing at room temperature for 3 hours. After the mixture had been allowed to stand at 5° for 16 hours, a precipitate had formed which was collected on a sintered glass filter. The precipitate was washed thoroughly with water and then dried *in vacuo* over phosphoric anhydride at room temperature. The light yellow powder weighed 2.67 gm. (94 per cent). The mercury derivative (IIa) was slightly soluble in dioxane, ethanol, and acetone, very slightly soluble in ethyl acetate, and insoluble in water.

$C_{16}H_{17}N_2O_4SClHg$. Calculated. N 4.92, S 5.63
569.5 Found. " 4.86, " 5.40

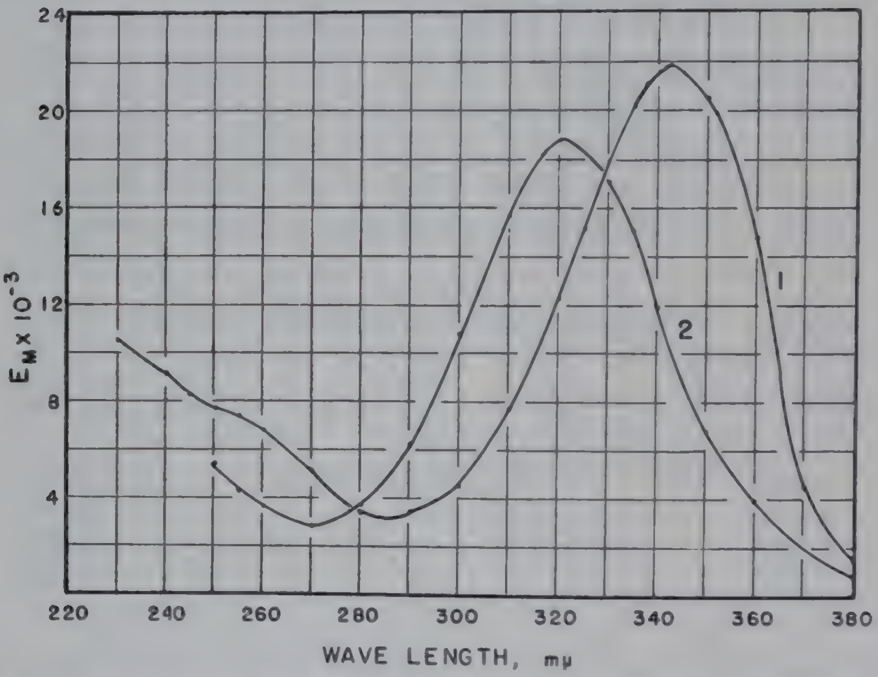


FIG. 2. Molar absorption spectra[1] of the mercury derivative (IIa) in 95 per cent ethanol (Curve 1) and in dioxane (Curve 2).

The molar absorption spectra of the mercury derivative (IIa) in 95 per cent ethanol (Curve 1) and in dioxane (Curve 2) are shown in Fig. 2.

Conversion of Mercury Derivative (IIa) to Amorphous D-*Benzylpenicillenic Acid*—A suspension of 2.40 gm. of the mercury derivative (IIa) in 30 cc. of water and 75 cc. of ethyl acetate was treated with hydrogen sulfide. The mixture was centrifuged and the ethyl acetate layer was filtered through diatomaceous earth (Filter-Cel). The filtrate was placed at −70° for 30 minutes to freeze out most of the water. The ice crystals were separated by rapid filtration and washed with 50 cc. of ethyl acetate at −70°. The combined ethyl acetate solutions were allowed to stand for 30 minutes over anhydrous $MgSO_4$. After the desiccant had been separated by gravity filtration, the ethyl acetate solution was concentrated *in vacuo* in the absence of air of ebullition to a volume of 30 cc. The concentrated ethyl acetate solution was added dropwise to 600 cc. of agitated hexane. The amorphous precipitate which formed turned to a gum when the mixture was allowed to stand for 2 hours at 5°. The supernatant liquid was decanted and the gum was dissolved in 30 cc. of chloroform. Upon dropwise addition of the chloroform solution to 500 cc. of hexane, a white, amorphous precipitate formed. The precipitate was filtered, washed well with hexane, and, while still moist with hexane, placed in a vacuum desiccator to dry under suction. The amorphous D-benzylpenicillenic acid weighed 0.85 gm. (60 per cent). This material possessed an absorption peak in 95 per cent ethanol at 322.5 mμ (E_M = 17,600). The specific rotation determined within 10 minutes after preparing the solution was $[\alpha]_D^{24} = +86°$ (0.47 per cent solution in 95 per cent ethanol).

DL-*Benzylpenicillenic Acid by Admixture of Natural* D- *with Synthetic* L-*Benzylpenicillenic Acid*—Natural D-benzylpenicillenic acid (0.70 gm.) (E_M = 17,600 at 322.5 mμ; $[\alpha]_D^{24} = +86°$) was admixed with 0.70 gm. of synthetic L-benzylpenicillenic acid (E_M = 17,800 at 322.5 mμ; $[\alpha]_D^{24} = -89°$). The mixture was treated by a procedure similar to that described above for the isolation of crystalline DL-benzylpenicillenic acid by admixture of the synthetic enantiomorphs. The crystalline DL-benzylpenicillenic acid isolated by this procedure weighed 0.283 gm. (21 per cent), m.p. 133–134° (with decomposition).

A 100 mg. sample of this product was recrystallized from 5 cc. of ethyl acetate. 50 mg. of DL-benzylpenicillenic acid were recovered; m.p. 137–139° (with decomposition); $[\alpha]_D^{23} = 0°$ (0.26 per cent solution in 95 per cent ethanol). There was no depression in the melting point upon admixture with DL-benzylpenicillenic acid prepared from DL-penicillamine (V) and the oxazolone (IV) (7). The molar absorption spectrum in 95 per cent ethanol is shown in Fig. 3.

$C_{16}H_{18}N_2O_4S$.	Calculated.	C 57.45, H 5.43, N 8.38
334.4	Found.	" 57.31, " 5.82, " 8.33

Infra-Red Absorption Measurements—The infra-red absorption spectra from 690 cm.$^{-1}$ to 3600 cm.$^{-1}$ of DL-benzylpenicillenic acid made by admixture of natural D- with synthetic L-benzylpenicillenic acid (upper curve) and of DL-benzylpenicillenic acid made by synthesis from DL-penicillamine (lower curve) are shown in Fig. 4. The absorption spectra were determined on samples of the crystalline compounds mulled in mineral oil between two sodium chloride plates. The measurements were made on a Perkin-Elmer infra-red spectrometer, model 12A, with a gain control to compensate for the energy distribution of the Globar source. It should be pointed out that the curves include absorption peaks due to mineral oil and to air as well as those due to benzylpenicillenic acid.

Rearrangement of Benzylpenicillenic Acid to Benzylpenicillin—Crystalline

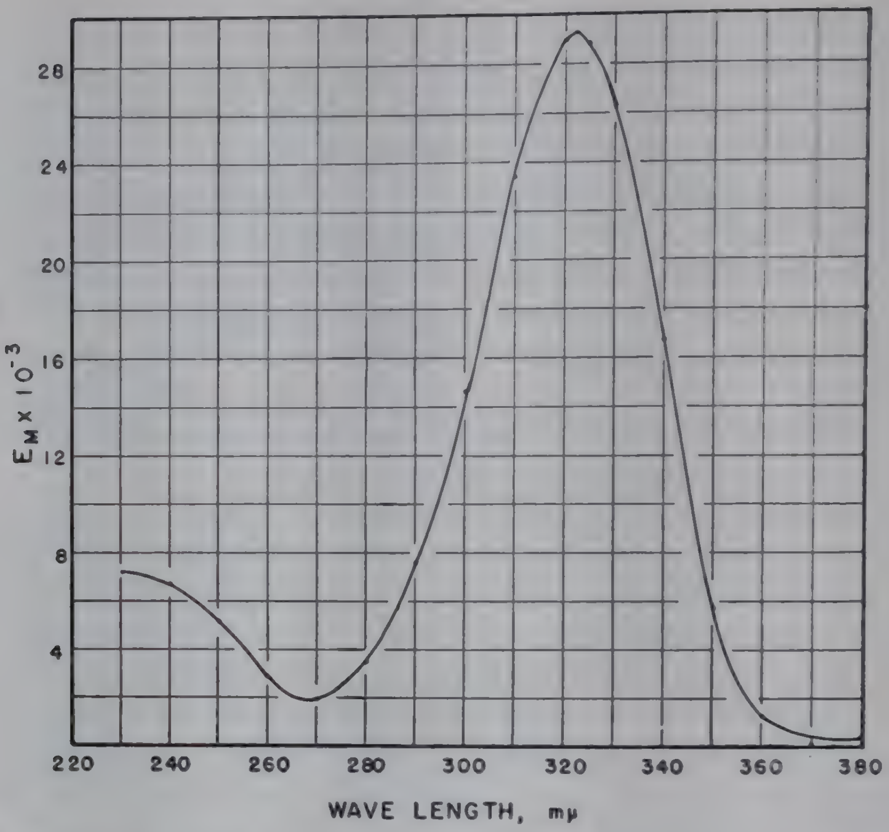


FIG. 3. Molar absorption spectrum[1] of crystalline DL-benzylpenicillenic acid in 95 per cent ethanol.

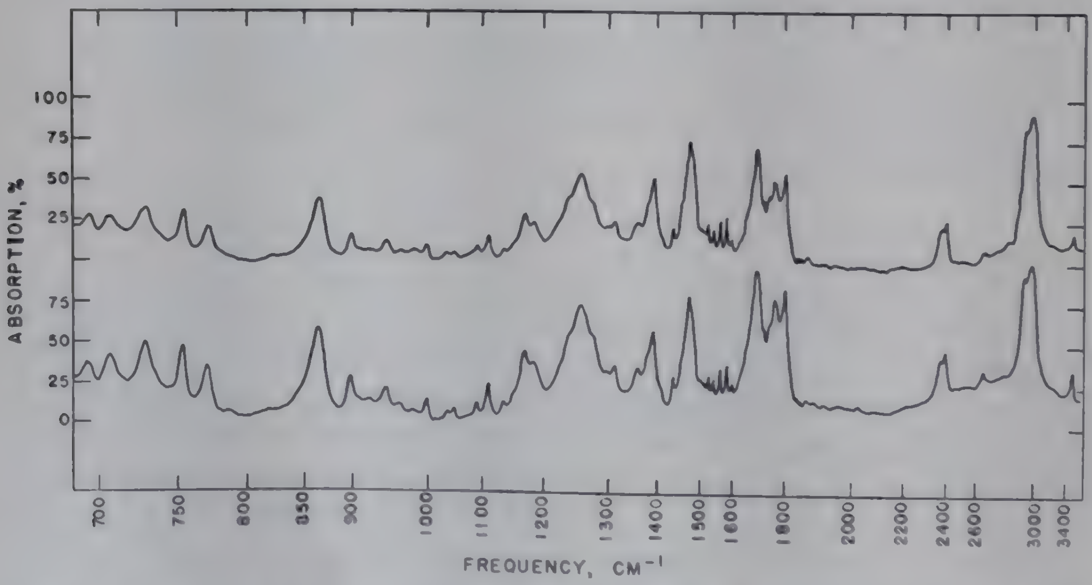


FIG. 4. Infra-red absorption spectra of crystalline DL-benzylpenicillenic acids: upper curve, DL-benzylpenicillenic acid prepared by admixture of natural D- with synthetic L-benzylpenicillenic acid; lower curve, DL-benzylpenicillenic acid synthesized from DL-penicillamine.

DL-benzylpenicillenic acid (30 mg.) prepared by admixture of natural D- with synthetic L-benzylpenicillenic acid was dissolved in 10 cc. of pyridine containing 6.5 mg. of pyridinium chloride per cc. Aliquots (1 cc.) of this solution were placed in a series of test-tubes. To one of these tubes about 0.05 cc. of triethylamine was added and the tube was placed in an ice bath. The rest of the tubes were placed in an oil bath at 120°. At noted time intervals a tube was removed from the oil bath, triethylamine was added to it, and the tube was cooled in the ice bath. The solvents were removed from each tube *in vacuo* at a bath temperature of 50°. The residues were moistened with 0.2 cc. of acetone and then dissolved in various amounts of 1 per cent phosphate buffer solution at pH 6. These buffer solutions were assayed against *Bacillus subtilis* ATCC 6051 by a modification of the method of Vincent and Vincent (9) with crystalline sodium benzylpenicillin as a standard. The results are shown in Curve 1, Fig. 5.

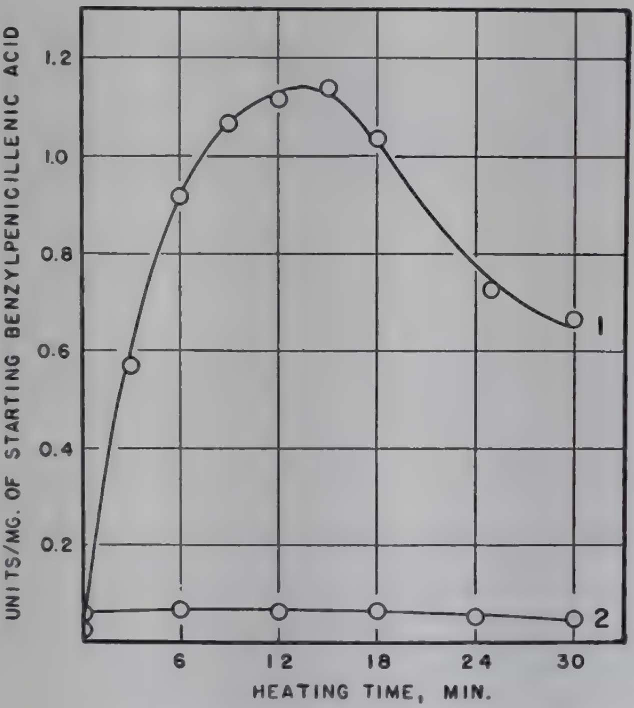


FIG. 5. Antibiotic activity produced by heating benzylpenicillenic acids in pyridine containing pyridinium chloride: Curve 1, crystalline DL-benzylpenicillenic acid in which the D moiety came from penicillin; Curve 2, synthetic, amorphous L-benzylpenicillenic acid.

Synthetic L-benzylpenicillenic acid was heated in pyridine and pyridinium chloride, and the products were prepared for assay under conditions similar to those described above. The results are shown in Curve 2, Fig. 5. It should be noted that this crude L-benzylpenicillenic acid possessed a slight amount of antibiotic activity (about 0.05 unit per mg.) when assayed at high concentration. However, since this antibiotic activity increased only very slightly, if at all, during the heating period, it was probably not due to the presence of a penicillin-like compound.

In another experiment, crystalline DL-benzylpenicillenic acid made by admixture of natural D with synthetic L acid was dissolved at a concentration of 3 mg. per cc. in pyridine containing 6.5 mg. of pyridinium chloride per cc. An identical solution was prepared from DL-benzylpenicillenic acid made by synthesis from DL-penicillamine (7). The two solutions were placed in an oil bath at 110° for 12 minutes, and then removed and prepared for assay as described above. The DL-benzylpenicillenic acid in which the D moiety arose from penicillin yielded 1.14 units of penicillin per mg. of starting DL-benzylpenicillenic acid, while the entirely synthetic DL-benzylpenicillenic acid gave rise to 1.19 units. Aliquots which had not been heated were also assayed. These showed no detectable activity when assayed at a concentration of 3 mg. per cc.

Rearrangement of Natural D-Benzylpenicillenic Acid to D-Benzylpenillic Acid—A solution of 75 mg. of amorphous, natural D-benzylpenicillenic acid in 1 cc. of methanol was seeded with a trace of D-benzylpenillic acid. The solution was allowed to stand at room temperature for 18 hours and then at 5° for 24 hours. Long, needle-like crystals of D-benzylpenillic acid separated; weight, 10.5 mg. (14 per cent); $[\alpha]_D^{24} = +490°$ (0.1 per cent solution in methanol) (6); $E_M = 6300$ at 237.5 mμ in 95 per cent ethanol (6). The melting point was determined on a sample which had been recrystallized by dissolving it in an equivalent amount of 0.1 N NaOH and then adding an equivalent amount of 0.1 N HCl. This sample of D-benzylpenillic acid melted at 180–185° (with decomposition).[5] The melting point was not lowered upon admixture with D-benzylpenillic acid prepared by rearrangement of benzylpenicillin in water at pH 2 (8).

Rearrangement of DL-Benzylpenicillenic Acid to DL-Benzylpenillic Acid—A methanolic solution (2 cc.) of 100 mg. of crystalline DL-benzylpenicillenic acid in which the D moiety arose from penicillin was seeded with a trace of DL-benzylpenillic acid. After the solution had been allowed to stand for 17 hours at room temperature and 24 hours at 5°, 18.2 mg. (18 per cent) of long, needle-like crystals separated. The DL-benzylpenillic acid had a specific rotation of $[\alpha]_D^{24} = 0°$ (0.1 per cent solution in methanol); $E_M = 5700$ at 240 mμ in 95 per cent ethanol; and m.p. 179–180° (with decomposition).[5] The melting point was not lowered upon admixture with DL-benzylpenillic acid prepared by synthesis from DL-penicillamine through the intermediate DL-benzylpenicillenic acid (7).

---

[5] In the determination of the melting points of the benzylpenillic acids reported in this paper, the compounds were placed in the bath at 170° and heated at a rate of 1.5° per minute at the melting point.

DL-*Benzylpenillic Acid by Admixture of Enantiomorphs*—D-Benzylpenillic acid and its enantiomorph were prepared from the corresponding D- and L-benzylpenicillenic acids by rearrangement in methanol (6). Each of the penillic acids was dissolved in 0.1 N NaOH solution so that the concentration was 30 mg. per cc. Equal volumes of the two solutions were mixed, and the resulting solution was made acid to Congo red paper with 0.1 N HCl. After the solution had stood at 5° overnight, it yielded white crystals, m.p. 177–178° (with decomposition). The melting point of this DL-benzylpenillic acid was not lowered upon admixture with racemic benzylpenillic acid (m.p. 178.5–179.5°) formed by rearrangement of DL-benzylpenicillenic acid (7).

The authors wish to thank Dr. Julian R. Rachele for aid with the infrared measurements, Miss Josephine E. Tietzman for carrying out the microanalyses, and Dr. Dorothy S. Genghof and Miss Mary R. Lloyd for performing the penicillin assays.

SUMMARY

D-Benzylpenicillenic acid synthesized from D-penicillamine hydrochloride and 2-benzyl-4-methoxymethylene-5(4)-oxazolone was shown to be identical with D-benzylpenicillenic acid prepared by rearrangement of D-benzylpenicillin (penicillin G).

Benzylpenicillin was converted to an antibiotically inactive product which was in turn reconverted in small yield to benzylpenicillin. Evidence was presented to show that this inactive compound was identical with D-benzylpenicillenic acid and that D-benzylpenicillenic acid was an intermediate in the synthesis of benzylpenicillin from D-penicillamine and 2-benzyl-4-methoxymethylene-5(4)-oxazolone.

Racemic benzylpenillic acid prepared by rearrangement of DL-benzylpenicillenic acid was shown to contain a D moiety identical with D-benzylpenillic acid prepared by rearrangement of D-benzylpenicillin.

BIBLIOGRAPHY

1. Committee on Medical Research, Office of Scientific Research and Development, Washington, and Medical Research Council, London, *Science*, **102**, 627 (1945).
2. du Vigneaud, V., Carpenter, F. H., Holley, R. W., Livermore, A. H., and Rachele, J. R., *Science*, **104**, 431 (1946).
3. Peck, R. L., and Folkers, K., in Clarke, H. T., Johnson, J. R., and Robinson, R., The chemistry of penicillin, Princeton, chapter 7, in press.
4. du Vigneaud, V., Wood, J. L., and Wright, M. E., in Clarke, H. T., Johnson, J. R., and Robinson, R., The chemistry of penicillin, Princeton, chapter 23, in press.
5. du Vigneaud, V., Carpenter, F. H., Holley, R. W., Livermore, A. H., and Rachele, J. R., in Clarke, H. T., Johnson, J. R., and Robinson, R., The chemistry of penicillin, Princeton, chapter 28, in press.

6. Holley, R. W., Carpenter, F. H., Livermore, A. H., and du Vigneaud, V., *Science*, **108**, 136 (1948).
7. Livermore, A. H., Carpenter, F. H., Holley, R. W., and du Vigneaud, V., *J. Biol. Chem.*, **175**, 721 (1948).
8. Cook, A. H., in Clarke, H. T., Johnson, J. R., and Robinson, R., The chemistry of penicillin, Princeton, chapter 6, in press.
9. Vincent, J. G., and Vincent, H. W., *Proc. Soc. Exp. Biol. and Med.*, **55**, 162 (1944).

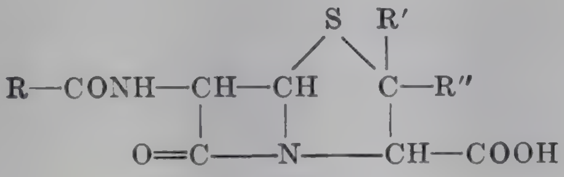
THE SYNTHESIS OF DL-β,β-DIETHYLCYSTEINE AND DL-β-ETHYL-β-METHYLCYSTEINE

By VINCENT du VIGNEAUD, GARDNER W. STACY,
AND DAVID TODD

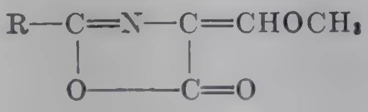
(*From the Department of Biochemistry, Cornell University Medical College, New York City*)

(Received for publication, July 30, 1948)

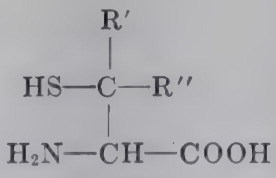
The various penicillins of natural origin that have so far been isolated (1–4) differ from one another only in the nature of the R group, as illustrated in the general formula (I) based on the β-lactam structure for penicillin. However, the results of chemical studies in this and other laboratories (5, 6) indicate that several penicillins in which various groups have been substituted at R′ and R″ (I) have been synthesized in minute yield, although as yet these substances have not been isolated in pure form. These penicillins were prepared by the condensation of an oxazolone (II) with the appropriate α-amino-β-mercapto acid (III).



(I)



(II)



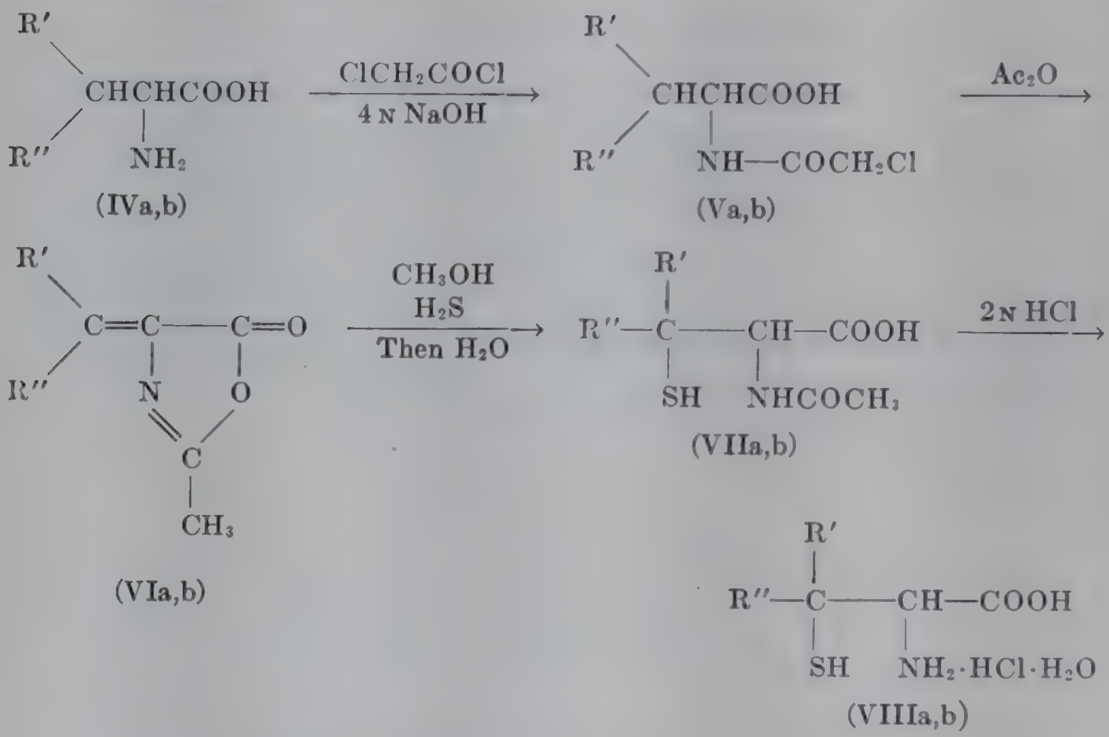
$$\begin{array}{c} R' \\ | \\ HS-C-R'' \\ | \\ H_2N-CH-COOH \end{array}$$

(III)

(IIIa) ($R' = R'' = C_2H_5$)
(IIIb) ($R' = C_2H_5$, $R'' = CH_3$)
(IIIc) ($R' = R'' = CH_3$)

For a study (7) of further variations of the penicillin molecule at R′ and R″, it was desired to prepare additional α-amino-β-mercapto acids for condensation with an appropriate oxazolone. The synthesis of two such compounds, namely DL-β,β-diethylcysteine (IIIa) and DL-β-ethyl-β-methylcysteine (IIIb), is the subject of the present paper. During the war-time studies on penicillin, the investigators at the Abbott Laboratories reported the preparation of β-ethyl-β-methylcysteine (IIIb) (8), but did not fully characterize the final product. Consequently the details of its preparation and isolation as the hydrochloride monohydrate (VIIIb) are included here.

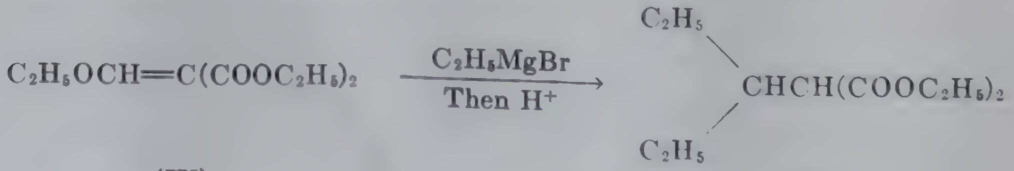
The series of reactions used for the synthesis of these two α-amino-β-mercapto acids was similar to that already developed for the synthesis of DL-penicillamine (IIIc) (8).



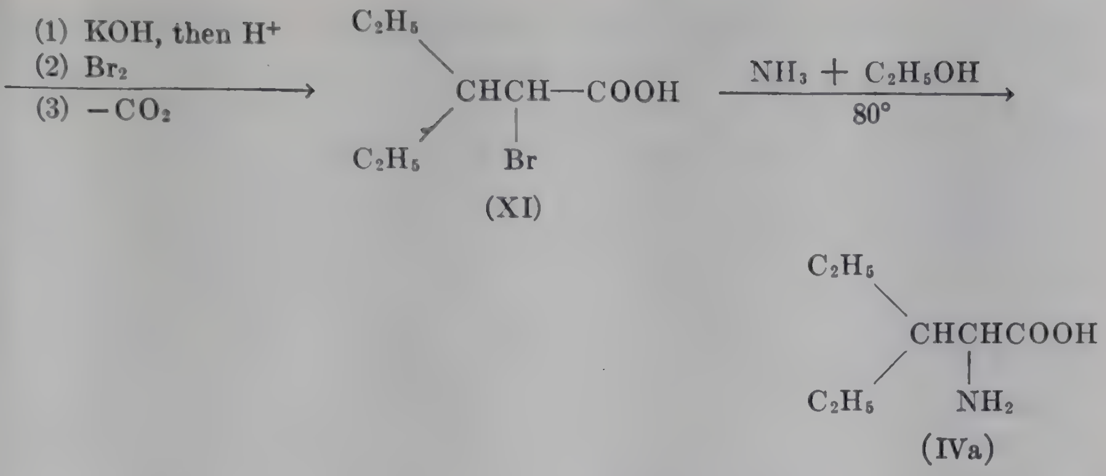
(IVa,b) (Va,b) (VIa,b) (VIIa,b) (VIIIa,b)

(a) (R′ = R″ = C_2H_5); (b) (R′ = C_2H_5, R″ = CH_3)

In the above reactions the commercially available DL-isoleucine (IVb) served as a starting compound for the synthesis of DL-β-ethyl-β-methylcysteine hydrochloride monohydrate (VIIIb). However, the DL-β,β-diethylalanine (IVa) needed as a starting compound for the preparation of DL-β,β-diethylcysteine hydrochloride monohydrate (VIIIa) had not been prepared previously. The method devised for the synthesis of the DL-β,β-diethylalanine is outlined in the accompanying equations.



(IX) (X)



(XI)

(IVa)

Diethyl (1-ethylpropyl)-malonate (X) was prepared in 80 per cent yield by the action of ethyl magnesium bromide with ethoxymethylenemalonic ester (IX) according to the procedure of Reynolds (9). The malonic ester derivative (X) was converted to β,β-diethylalanine (IVa) by the malonic ester synthesis of amino acids (10). In this series of reactions the saponification of the malonic ester, bromination of the resulting acid, and decarboxylation of the α-bromo acid proceeded smoothly. However, when aqueous ammonia was used to aminate the α-bromo-β-ethylvaleric acid, which was not obtained in pure state, difficulty was encountered in the isolation of the amino acid from the reaction mixture. This difficulty was overcome by carrying out the amination in ethanolic ammonia.

EXPERIMENTAL[1]

Diethyl (1-Ethylpropyl)-malonate—Ethyl magnesium bromide was prepared by the addition of 272 gm. of ethyl bromide in 300 cc. of dry ether to 61 gm. of magnesium in 400 cc. of dry ether. After the addition of the ethyl bromide had been completed, the reaction mixture was heated under gentle reflux. Then 216 gm. of ethoxymethylenemalonic ester in 150 cc. of ether were added over a period of 2 hours. Cooling of the reaction mixture in a water bath was necessary during this addition. After the reaction mixture had cooled to room temperature, it was poured slowly onto a mixture of 215 cc. of 12 N HCl and 1 kilo of ice. The ether layer was separated, and the aqueous layer was shaken with three 200 cc. portions of ether. After the combined ether layers had been dried over anhydrous $MgSO_4$, the ether was removed and the residue was distilled. The diethyl (1-ethylpropyl)-malonate distilled at 112–113° and amounted to 183 gm.

---

[1] All the melting points are corrected and are capillary melting points unless otherwise specified.

or 80 per cent of the theoretical amount based on ethoxymethylenemalonic ester.

α-Bromo-β-ethylvaleric Acid—171 gm. of KOH were dissolved in 150 cc. of water and the solution was heated to 100°. To this solution, diethyl (1-ethylpropyl)-malonate (183 gm.) was added dropwise at first and then more rapidly as the reaction got under way. After the addition was complete, the reaction mixture was heated at 100° with stirring for 5 hours.

The contents of the flask were transferred to a beaker, cooled to 15°, and acidified by the addition of 274 cc. of 12 N HCl. A precipitate which formed after the addition of about 170 cc. of acid disappeared upon addition of the rest of the acid. The aqueous solution was shaken with three 200 cc. portions of ether and the combined ether layers were dried over $CaCl_2$. The ether solution was concentrated to a volume of about 400 cc. and bromine (37.8 cc., 113 gm.) was added. The first 3 to 5 cc. of bromine were added in one portion and the mixture was stirred until the color had disappeared. The remainder of the bromine was added dropwise over a period of about 1 hour. After the addition of bromine was complete, 140 cc. of water were added slowly so as not to produce foaming. The ether layer was separated and the aqueous layer was shaken with a 100 cc. portion of ether. The ether layers were combined, the ether was removed, and the residue was subjected to decarboxylation by heating under a reflux at 140° for 2 hours. The residue was distilled at 5 mm. and the crude α-bromo-β-ethylvaleric acid was collected in two fractions (b.p. 106–125°, 30.1 gm., and b.p. 125–141°, 93.7 gm.). The total weight of these two fractions corresponded to 74 per cent of the theoretical amount based on diethyl (1-ethylpropyl)-malonate.

DL-*β,β-Diethylalanine*—A mixture of 30 gm. of the crude α-bromo-β-ethylvaleric acid (b.p. 125–141°) and 75 cc. of absolute ethanol was cooled to −70° and 45 gm. of liquid ammonia were added. The mixture was heated in an autoclave to 80° over a period of 5 hours. The ethanol and ammonia were then removed by a stream of air and the residue was washed with ether. Although the product consisted of a mixture of the desired amino acid and NH_4Br, it was suitable for our use without further purification. The weight of the mixture amounted to 34.8 gm. When the fraction of α-bromo-β-ethylvaleric acid boiling at 106–125° at 5 mm. was used, a lower yield of product was obtained.

A sample was purified for analysis by recrystallization from 70 per cent ethanol. On the hot stage the crystals of DL-β,β-diethylalanine changed from prisms to needles at 170–185° and melted at 245–249° (micro).

$C_7H_{15}O_2N$.	Calculated.	C 57.9, H 10.41, N 9.65
145.2	Found.	" 57.7, " 10.23, " 9.47

*N-Chloroacetyl-*DL*-β,β-diethylalanine*—14.4 gm. of crude β,β-diethylalanine were dissolved in 16 cc. of 4 N NaOH and 30 cc. of water. While the solution was cooled in an ice bath, 14.3 gm. of chloroacetyl chloride and 55 cc. of 4 N NaOH were added dropwise with stirring. Then the solution was acidified with 6.3 cc. of 12 N HCl, causing the precipitation of the product. The crude N-chloroacetyl-DL-β,β-diethylalanine (m.p. 122–123.5°) weighed 8.2 gm.

27.0 gm. from several runs were dissolved in 100 cc. of ethanol and 250 cc. of water. The hot solution was treated with 1 gm. of norit and filtered. The N-chloroacetyl-DL-β,β-diethylalanine crystallized from the cooled solution, m.p. 127–129°.

$C_9H_{16}O_3ClN$.	Calculated.	C 48.8, H 7.28, N 6.32
221.7	Found.	" 48.9, " 7.50, " 6.36

2-Methyl-4-(1′-ethylpropylidene)-5(4)-oxazolone—27.2 gm. of recrystallized N-chloroacetyl-β,β-diethylalanine and 40 cc. of acetic anhydride were heated in an oil bath at 60–70° for a period of 2 hours. At the end of this time the acetic anhydride was removed under reduced pressure (water pump) at 60° and the oxazolone was distilled at 54–55° at 0.01 to 0.05 mm. The yield was 11.1 gm. or 54 per cent of the theoretical amount.

For purposes of characterization the oxazolone was converted to α-acetamido-β,β-diethylacrylic acid by heating the oxazolone in an excess of water. The acrylic acid derivative was recrystallized from ethyl acetate to give prisms, m.p. 178–178.5° (with decomposition).

$C_9H_{15}O_3N$.	Calculated.	C 58.4, H 8.16, N 7.57
185.2	Found.	" 58.1, " 8.37, " 7.74

α-Acetamido-β,β-diethylacrylamide was also readily obtained by dissolving 0.2 gm. of the oxazolone in 10 cc. of 10 per cent NH_4OH. After the solution had cooled a crystalline precipitate formed, m.p. 198–214°. This was recrystallized from ethanol, m.p. 220–227°.

$C_9H_{16}O_2N_2$ (184.2). Calculated, N 15.21; found, N 15.33

*N-Acetyl-*DL*-β,β-diethylcysteine*—To 48.5 cc. of methanol was added 0.3 gm. of sodium and the resulting solution was saturated with H_2S. 11.0 gm. of 2-methyl-4-(1′-ethylpropylidene)-5(4)-oxazolone were added and H_2S was passed through the solution for 12 hours. The reaction mixture was acidified with 1.1 cc. of 12 N HCl and the methanol was removed by distillation under reduced pressure. The crystalline residue was dissolved in a mixture of 55 cc. of methanol and 225 cc. of water and treated with 1 gm. of charcoal (norit). The N-acetyl-DL-β,β-diethylcysteine (m.p. 158–161°) recovered from the solution weighed 8.9 gm. or 62 per cent of the theoretical amount. After a sample of the crude compound had been recrystallized twice from aqueous ethanol, it possessed a melting point of 167–168°.

$C_9H_{17}O_3NS$. Calculated. C 49.3, H 7.82, S 14.62
219.3 Found. " 49.3, " 7.80, " 14.93

DL-β,β-*Diethylcysteine Hydrochloride Monohydrate*—7.2 gm. of the N-acetyl-DL-β,β-diethylcysteine were heated under a reflux with 100 cc. of 2 N HCl for 16 hours. After the reaction mixture had been concentrated to a volume of about 30 cc., a crystalline precipitate formed which weighed 2.68 gm., representing 35 per cent of the theoretical amount. A 100 mg. sample was recrystallized twice from 5 cc. of 12 N HCl. The twice recrystallized material was dissolved in 0.2 cc. of absolute ethanol and to this were added 2 cc. of absolute ether. The crystals thus obtained had a capillary melting point of 126–127°. However, when the crystals were heated slowly on the hot stage, they changed from prisms to needles at 152° and melted at 176–177° (micro).

$C_7H_{15}O_2NS \cdot HCl \cdot H_2O$. Calculated. C 36.3, H 7.83, Cl 15.32
231.7 Found. " 36.4, " 7.97, " 15.38

2-Methyl-4-(sec-butylidene)-5(4)-oxazolone—A mixture of 69.6 gm. of N-chloroacetyl-DL-isoleucine (11) and 110 cc. of acetic anhydride was agitated by a stream of nitrogen while being heated at 55–60° for 2 hours. After the acetic anhydride had been removed by distillation under reduced pressure (water pump), the residue was distilled at 0.01 to 0.2 mm. and the product distilling at 50–61° was collected. The yield of oxazolone was 41.0 gm. or 79 per cent of the theoretical amount. It was found advisable to use this material immediately for the preparation of N-acetyl-DL-β-ethyl-β-methylcysteine.

The oxazolone was characterized by conversion to α-acetamido-β-ethyl-β-methylacrylic acid (m.p. 174–175°) by heating the oxazolone in an excess of water.

$C_8H_{13}O_3N$. Calculated. C 56.1, H 7.65, N 8.18
171.2 Found. " 56.2, " 7.68, " 8.01

*N-Acetyl-*DL*-β-ethyl-β-methylcysteine*—1.15 gm. of sodium were dissolved in 190 cc. of methanol and the resulting solution was saturated with H_2S. 41 gm. of 2-methyl-4-(*sec*-butylidene)-5(4)-oxazolone were dissolved in 55 cc. of methanol and this solution was added to the sodium methylate solution. H_2S was passed through the reaction mixture for 16 hours. The mixture was acidified with 4.5 cc. of 12 N HCl and the methanol was removed by distillation under reduced pressure. The residue was crystallized from a mixture of 150 cc. of water and 5 cc. of methanol. This crude product (m.p. 138–138.5°) weighed 44.1 gm. or 80 per cent of the theoretical amount. After the crude product had been treated with charcoal (norit) and crystallized from water, the N-acetyl-DL-β-ethyl-β-methylcysteine possessed a melting point of 144–146.5° and was suitable for conversion to the amino acid. A sample prepared for analysis by two recrystallizations from water had a melting point of 144–145°.

$C_8H_{15}O_3NS$.	Calculated.	C 46.8, H 7.37, S 15.62
205.3	Found.	" 46.6, " 7.44, " 15.86

DL-*β-Ethyl-β-methylcysteine Hydrochloride Monohydrate*—6.1 gm. of the recrystallized N-acetyl-β-ethyl-β-methylcysteine were heated under a reflux with 85 cc. of 2 N HCl for 16 hours. The volume of the reaction mixture was concentrated to about 20 cc. and the crystalline product was collected. The yield amounted to 2.41 gm. or 38 per cent of the theoretical amount. The β-ethyl-β-methylcysteine hydrochloride monohydrate was purified by recrystallization from 12 N HCl. As in the case of the β,β-diethylcysteine hydrochloride monohydrate, a difference was noted between the capillary melting point and that obtained on the hot stage. The capillary melting point was 117–119°, while that obtained on the hot stage was 169–170° (micro).

$C_6H_{13}O_2NS \cdot HCl \cdot H_2O$.	Calculated.	C 33.1, H 7.41, Cl 16.29
217.7	Found.	" 33.1, " 7.74, " 16.29

The authors wish to express their appreciation to Miss Josephine E. Tietzman for the microanalyses reported in this paper.

SUMMARY

The synthesis of DL-β,β-diethylalanine and its use in the preparation of DL-β,β-diethylcysteine hydrochloride monohydrate have been described. In addition, details have been presented for the synthesis of DL-β-ethyl-β-methylcysteine hydrochloride monohydrate from DL-isoleucine.

BIBLIOGRAPHY

1. Committee on Medical Research, Office of Scientific Research and Development, Washington, and Medical Research Council, London, *Science*, **102**, 627 (1945).
2. Editorial Board of the monograph on the chemistry of penicillin, *Science*, **106**, 503 (1947).
3. Clarke, H. T., Johnson, J. R., and Robinson, R., The chemistry of penicillin, Princeton, in press.
4. Behrens, O. K., *cf.* (3) chapter 19.
5. du Vigneaud, V., Wood, J. L., and Wright, M. E., *cf.* (3) chapter 23.

6. du Vigneaud, V., Carpenter, F. H., Holley, R. W., Livermore, A. H., and Rachele, J. R., *Science*, **104**, 431 (1946).
7. Carpenter, F. H., Stacy, G. W., Genghof, D. S., Livermore, A. H., and du Vigneaud, V., *J. Biol. Chem.*, **176**, 915 (1948).
8. Crooks, H. M., *cf.* (3) chapter 16.
9. Reynolds, G. P., *Am. Chem. J.*, **44**, 305 (1910).
10. Marvel, C. S., in Organic syntheses, New York, **21**, 60 (1941).
11. Abderhalden, E., Hirsch, P., and Schuler, J., *Ber. chem. Ges.*, **42**, 3394 (1909).

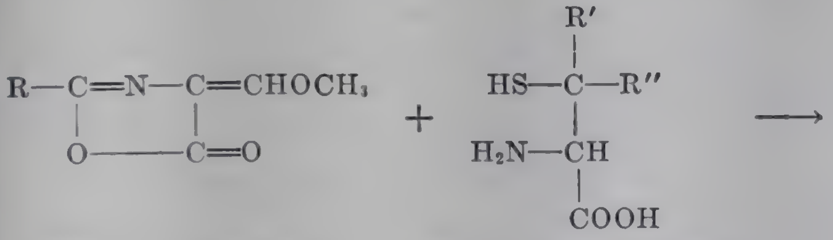
THE PREPARATION AND ANTIBACTERIAL PROPERTIES OF THE CRUDE SODIUM SALTS OF SOME SYNTHETIC PENICILLINS*

By FREDERICK H. CARPENTER, GARDNER W. STACY,
DOROTHY S. GENGHOF, ARTHUR H. LIVERMORE,
AND VINCENT DU VIGNEAUD

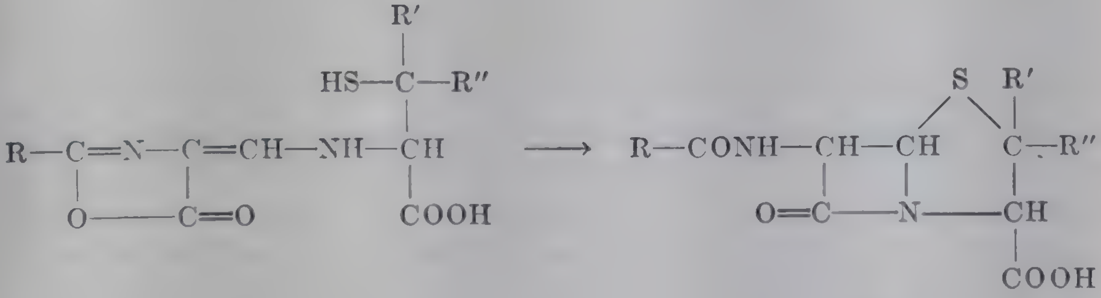
(*From the Department of Biochemistry, Cornell University Medical College, New York City*)

(Received for publication, July 30, 1948)

In previous communications (1–3) it has been demonstrated that the antibiotic activity (4) produced by heating D-penicillamine (II) hydrochloride hydrate with 2-benzyl-4-methoxymethylene-5(4)-oxazolone (I) in pyridine is due to the synthesis of benzylpenicillin. Because of these results there can be little doubt that, when oxazolones substituted with other groups in the 2 position are condensed with penicillamine hydrochloride in pyridine, the antibiotic activity (1, 4) produced is due to the synthesis of penicillins differing from benzylpenicillin in the nature of the



(I) (R = $C_6H_5CH_2$) (II) (R′ = R″ = CH_3)



(III) (IV)

(IIIa) (R = $C_6H_5CH_2$, R′ = R″ = CH_3)
(IIIb) (R = C_6H_5, R′ = R″ = CH_3)
(IIIc) (R = $C_6H_5CH{=}CH$, R′ = R″ = CH_3)

---

* This work was supported in part by a research grant from the National Institute of Health.

R group, as illustrated on the basis of the β-lactam structure (IV). Moreover, the antibiotic activity (1, 4) produced by the condensation of the oxazolone (I) with α-amino-β-mercapto acids other than D-penicillamine must be due to the synthesis of analogues of penicillin (IV) differing from the known varieties in the nature of the groups R′ and R″.

The results of studies (3, 5, 6) on the mechanism of synthesis of benzylpenicillin from 2-benzyl-4-methoxymethylene-5(4)-oxazolone (I) and D-penicillamine (II) have proved beyond reasonable doubt that D-benzylpenicillenic acid (IIIa) is an intermediate in the synthesis of benzylpenicillin. Since these studies were hindered by difficulties in obtaining this intermediate compound in crystalline form, it seemed advisable to introduce other oxazolones or other α-amino-β-mercapto acids into the investigation with the thought in mind that their use might result in the production of crystalline intermediate compounds. Some justification for this reasoning was afforded by the finding that the substitution of DL-penicillamine for D-penicillamine in the condensation with the oxazolone (I) in pyridine containing triethylamine resulted in the production of a crystalline intermediate compound, identified as DL-benzylpenicillenic acid (IIIa) (5, 6).

Although the mold produces a number of different penicillins (7), especially in the presence of specific precursors (8, 9), all of the penicillins thus far isolated from the mold cultures have contained the D-penicillamine moiety and have differed from one another only in the nature of the R group (IV). It was of interest, therefore, to prepare penicillins in which the D-penicillamine moiety was replaced by other α-amino-β-mercapto acids. Penicillins of this type could be prepared in crude form by the substitution of various α-amino-β-mercapto acids for D-penicillamine in the synthetic reaction. These crude penicillins could then be subjected to biological testing to determine whether or not the antibacterial properties of penicillin could be markedly changed by the replacement of the penicillamine moiety with various α-amino-β-mercapto acids.

In the present communication a report is made of the synthesis and antibacterial properties of penicillins obtained by condensation of 2-benzyl-4-methoxymethylene-5(4)-oxazolone (I) (10) with each of the following α-amino-β-mercapto acids: DL-β-methylcysteine, Isomer A (11); DL-β-methylcysteine, Isomer B (11); DL-β,β-diethylcysteine hydrochloride hydrate (12); and DL-β-ethyl-β-methylcysteine hydrochloride hydrate (12, 13). In addition, the synthesis and antibiotic activities of penicillins produced by condensation of D-penicillamine (II) hydrochloride hydrate (13) with either 2-phenyl-4-ethoxymethylene-5(4)-oxazolone (4, 10) or 2-styryl-4-ethoxymethylene-5(4)-oxazolone (4, 10) were studied.

The two-step reaction (1, 3) was used for the synthesis of these penicillins. In the first step the oxazolone was condensed with the α-amino-β-mercapto acid in aqueous pyridine containing triethylamine to give the intermediate penicillenic acid (III). In only one instance, the case of the D-styrylpenicillenic acid (IIIc), was the penicillenic acid obtained in crystalline form. In all the other cases, the penicillenic acids were obtained in the form of amorphous solids. The ultraviolet absorption spectra of the various penicillenic acids were determined. It was found (Table I) that all of the penicillenic acids made by condensation of 2-benzyl-4-methoxymethylene-5(4)-oxazolone (I) with various α-amino-β-mercapto acids possessed nearly identical absorption curves which, like that of crystalline DL-benzylpenicillenic acid (IIIa) (5, 6), had their main absorption peak at about 320 mμ. On the other hand, the D-phenylpenicillenic acid (IIIb) had its main absorption peak at 355 mμ, while the crystalline D-styrylpenicillenic acid (IIIc) had its main peak at 374 mμ (Fig. 1).

In the second step of the synthesis, the various penicillenic acids (III) were converted in small yield to the corresponding penicillins by heating the penicillenic acids at a concentration of 100 mg. per cc. in pyridine containing pyridinium chloride. Preliminary experiments were performed to determine the optimum heating time for obtaining the maximum yield of activity from the rearrangement of each penicillenic acid. An optimum time of 9 minutes was found for all of the penicillenic acids in which R′ and R″ (III) were both alkyl groups (Table I). However, under the same conditions the optimum heating time was increased to about 20 minutes when one alkyl group at either R′ or R″ was replaced by hydrogen. On the other hand, the substitution of various groups at R (III) did not appear to affect the optimum heating time. It should be pointed out in this connection that the optimum heating time for the rearrangement of a particular penicillenic acid is dependent on the concentration of the penicillenic acid as well as the concentration of the hydrogen chloride in the pyridine (3).

The crude products, containing minute amounts of the various penicillins, were converted to their sodium salts for biological testing. These sodium salts, obtained as amorphous solids, possessed an antibiotic activity of 0.015 to 0.50 unit per mg., depending upon the particular penicillin, when assayed against *Staphylococcus aureus* H with crystalline sodium benzylpenicillin as a standard. The antibiotic activity of each penicillin was also determined quantitatively on two other organisms, *Bacillus subtilis* (ATCC 6051) and *Vibrio metchnikovii* (ATCC 7708). The ratios of the antibiotic activities found on each of these two organisms to that found on *Staphylococcus aureus* were calculated (Table I). It is interesting to note that each of the new synthetic penicillins could be distinguished from each other by the differences in their effect on these three organisms.

The synthetic penicillins were also tested for antibiotic activity against

TABLE I

Preparation of Crude Penicillenic Acids and Corresponding Sodium Penicillins

5(4)-Oxazolones	α-Amino-β-mercapto acids	Crude penicillenic acids			Optimum heating time	Antibiotic activity of crude synthetic penicillin preparations		
		Yield	Absorption peaks			*S. aureus*	Ratios of antibiotic activity	
			Wave-length	$E_M \times 10^{-3}$*			*B. subtilis* / *S. aureus*	*V. metchnikovii* / *S. aureus*
		per cent	*mμ*		*min.*	*units per mg.*†		
2-Benzyl-4-methoxymethylene-	DL-β-Methylcysteine, Isomer A	68	320 240	18.6 4.5	20	0.26	0.82	0.45
"	DL-β-Methylcysteine, Isomer B	93	320 240	18.7 4.1	20	0.11	1.09	1.91
"	DL-β,β-Diethylcysteine·HCl-H_2O	86	320	14.7	9	0.42	1.13	0.58
"	DL-β-Ethyl-β-methylcysteine·HCl·H_2O	45	320	13.1	9	0.47	1.14	1.27
"	D-Penicillamine-HCl·H_2O	75	320 240	20.0 5.1	9			
2-Phenyl-4-ethoxymethylene-	D-Penicillamine-HCl·H_2O	95	355 285 240	29.0 6.2 10.5	9	0.018	0.72	5.17
2-Styryl-4-ethoxymethylene-	D-Penicillamine-HCl·H_2O	98	374 280	40.0 13.0	9	0.014	0.50	0.96

* E_M is the molar absorption coefficient and is equal to D/cl where D is log I_0/I, c is the concentration in moles per liter, and l is the thickness of cell in cm.

† Experiments (1, 4, 6) with D-, L-, and DL-penicillamine have shown that only the D form gives rise to antibiotic activity in the synthetic reaction. Therefore one would expect that the antibiotic activity produced by the racemic α-amino-β-mercapto acids used here would represent only one-half the activity obtainable from the pure D forms.

seven microorganisms which were resistant to the action of benzylpenicillin. None of the crude sodium penicillins when tested at a concentration of 20 mg. per cc. (0.3 to 10 *Staphylococcus aureus* units per cc., depending on the penicillin) inhibited the growth of these particular organisms. Therefore it appears that substitution of the methyl groups of benzylpenicillin at R′ and R″ (IV) by various other groups did not appreciably change the action of penicillin on the resistant bacteria. Also, the crude synthetic penicillins in which the benzyl group of benzylpenicillin at R (IV) was replaced by a phenyl or styryl group did not show marked antibiotic activity against the seven resistant organisms.

The isolation of D-styrylpenicillenic acid in crystalline form made it possible to corroborate other evidence (5, 6) that penicillenic acid is an intermediate in the synthesis of penicillin from the condensation of various oxazolones with α-amino-β-mercapto acids. The ability of D-styrylpenicillenic acid (IIIc) to produce a small amount of antibiotic activity upon heating it in pyridine containing pyridinium chloride was retained through several crystallizations of the compound. Furthermore, it was possible to demonstrate (Table II) that various fractions obtained from the recrystallization of D-styrylpenicillenic acid (IIIc) gave rise to antibiotic activity in an amount proportional to the D-styrylpenicillenic acid present in these fractions.

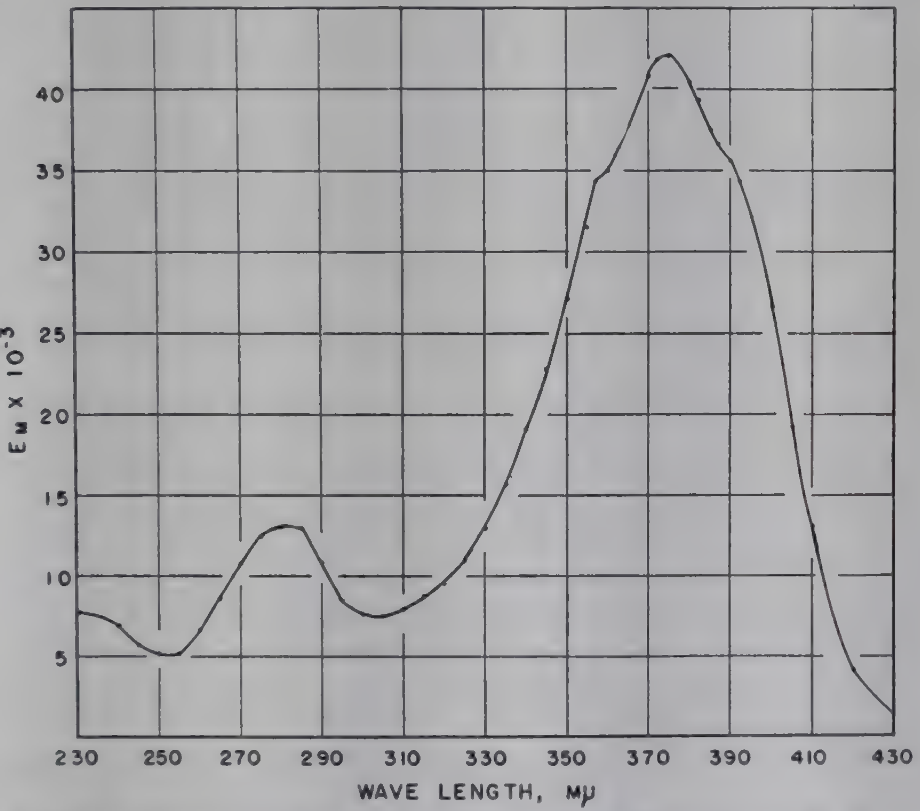


FIG. 1. Molar absorption spectrum of D-styrylpenicillenic acid in 95 per cent ethanol.

EXPERIMENTAL

Preparation of Penicillenic Acids—0.011 mole of the α-amino-β-mercapto acid hydrochloride hydrate was dissolved in 2.5 cc. of water or, if the free α-amino-β-mercapto acid was used, 0.011 mole of the compound was dissolved in 2.5 cc. of water containing an equivalent amount of HCl. The aqueous solution was cooled in an ice bath. The oxazolone (0.0102 mole) was dissolved in 10 cc. of pyridine, the solution was cooled to 0°, and 2.5 cc. of redistilled triethylamine were added. (In the case of DL-β,β-diethylcysteine and of DL-β-ethyl-β-methylcysteine, it was found necessary to use approximately 3 times as much water to dissolve these amino acids. Therefore, in the preparation of the oxazolone solutions, the volume of pyridine was increased in a proportional amount.) The cold oxazolone solution was added to the cold solution of the α-amino-β-mercapto acid. After the resulting mixture had been allowed to stand for 10 minutes in an ice bath, it was diluted with 125 cc. of ice-cold chloroform. The resulting mixture was immediately shaken for 1 minute with 125 cc. of ice-cold 2 M H_3PO_4. The chloroform layer was dried at 0° over anhydrous Na_2SO_4 and was then concentrated to dryness *in vacuo* in the absence of air of ebullition at a bath temperature of 30°.

The residue was dissolved in 22 cc. of chloroform and the solution was added dropwise to 450 cc. of agitated hexane. The resulting precipitate of a penicillenic acid was collected on a filter, washed well with hexane, and, while still moist with hexane, placed in a vacuum desiccator to dry under suction. If the resulting amorphous penicillenic acid was not used immediately, it was stored under anhydrous conditions at −10°. The yields of crude penicillenic acids, calculated on the basis of the amount of oxazolone used, and the positions of the absorption peaks in the ultraviolet region are recorded in Table I.

Crystalline D-*Styrylpenicillenic Acid*—D-Penicillamine hydrochloride hydrate (2.24 gm.) and 2.5 gm. of 2-styryl-4-ethoxymethylene-5(4)-oxazolone were condensed under conditions similar to those described above. When the dried chloroform solution of D-styrylpenicillenic acid was concentrated to 25 cc., a crystalline precipitate separated. The crude crystalline D-styrylpenicillenic acid weighed 1.55 gm., representing 45 per cent of the theoretical amount from the starting oxazolone, and possessed a melting point of 127–130° (with decomposition). After two recrystallizations from chloroform-hexane, the yellow crystals of the D-styrylpenicillenic acid melted at 146.5–148° (with decomposition) and had a specific rotation of $[\alpha]_D^{21} = -517°$ for a 0.3 per cent solution in chloroform.

$C_{17}H_{18}O_4N_2S$.	Calculated.	C 58.9, H 5.24
346.4	Found.	" 58.5, " 5.54

The molar absorption curve in the ultraviolet region of D-styrylpenicillenic acid in 95 per cent ethanol is shown in Fig. 1.

Determination of Optimum Heating Time for Conversion of Penicillenic Acids to Penicillins—The penicillenic acid was dissolved so as to give a solution with a concentration of 100 mg. per cc. in pyridine containing 6.5 mg. of pyridinium chloride per cc. An aliquot (0.1 to 0.2 cc.) was removed and added to about 0.05 cc. of triethylamine, and the resulting solution was cooled in an ice bath. The remainder of the original solution was placed in an oil bath preheated to 130°. At 3 or 4 minute intervals aliquots were removed, added to triethylamine, and cooled in an ice bath. The solvents were removed *in vacuo* from each aliquot in a water bath at 50°. The residues were moistened with 0.2 cc. of acetone and then dissolved in various amounts of 1 M phosphate buffer solution at pH 7. These buffer solutions were assayed against *Staphylococcus aureus* H by an agar diffusion method (14, 15) with crystalline sodium benzylpenicillin as a standard. The optimum heating times as determined by this method for the rearrangement of the various penicillenic acids are shown in Table I. The curves obtained by plotting the yield of activity against the heating time for D-styrylpenicillenic acid (broken line) and for the penicillenic acid made by the condensation of Isomer A of DL-β-methylcysteine with 2-benzyl-4-methoxymethylene-5(4)-oxazolone (solid line) are shown in Fig. 2.

Preparation of Crude Sodium Penicillins for Biological Testing—The penicillenic acids were rearranged in hot pyridine-pyridinium chloride in amounts no larger than 1 gm. The penicillenic acid (1 gm.) was dissolved in 10 cc. of pyridine containing 6.5 mg. of pyridinium chloride per cc. The resulting solution, contained in a 20 × 150 mm. test-tube, was placed in a large oil bath preheated to 130°. After the pyridine began to boil, the position of the tube in the bath was adjusted so that the pyridine refluxed gently from the sides of the tube. The reaction mixture was kept in the oil bath for the optimum length of time, as given in Table I. At the end of the heating period, 1 cc. of triethylamine was added to the reaction mixture and the resulting solution was cooled in an ice bath.

Two of the 1 gm. reaction mixtures were combined and the pyridine was removed under reduced pressure at 50° in such a manner that it took no longer than 8 minutes to concentrate the combined solutions to a gummy residue. The residue from 2 gm. of starting penicillenic acid was dissolved in 100 cc. of ice-cold chloroform and the resulting solution was shaken with 100 cc. of ice-cold 2 M phosphate buffer at pH 1.6 (prepared by admixture of equal volumes of 2 M H_3PO_4 and 2 M NaH_2PO_4). The chloroform layer was dried over anhydrous Na_2SO_4 at 0° and filtered from the desiccant, and the filtrate was diluted to 150 cc. with chloroform. A small amount of 1 N NaOH was added to 150 cc. of water and this solution was shaken with the chloroform solution. The pH of the aqueous layer was then determined. Additional alkali was added in discrete portions and the shaking with the chloroform solution was repeated until the pH of the aqueous layer reached 7 to 7.5. This procedure generally caused emulsions to form which were broken by centrifugation. The aqueous layer was immediately frozen and the water was removed by lyophilization. The resulting amorphous sodium salt was dried to constant weight in a vacuum desiccator over P_2O_5. This sodium salt which contained small amounts of the synthetic sodium penicillin was used for the biological testing. The weights of the crude sodium salts ranged from 50 to 70 per cent of the weights of the penicillenic acids used as starting material.

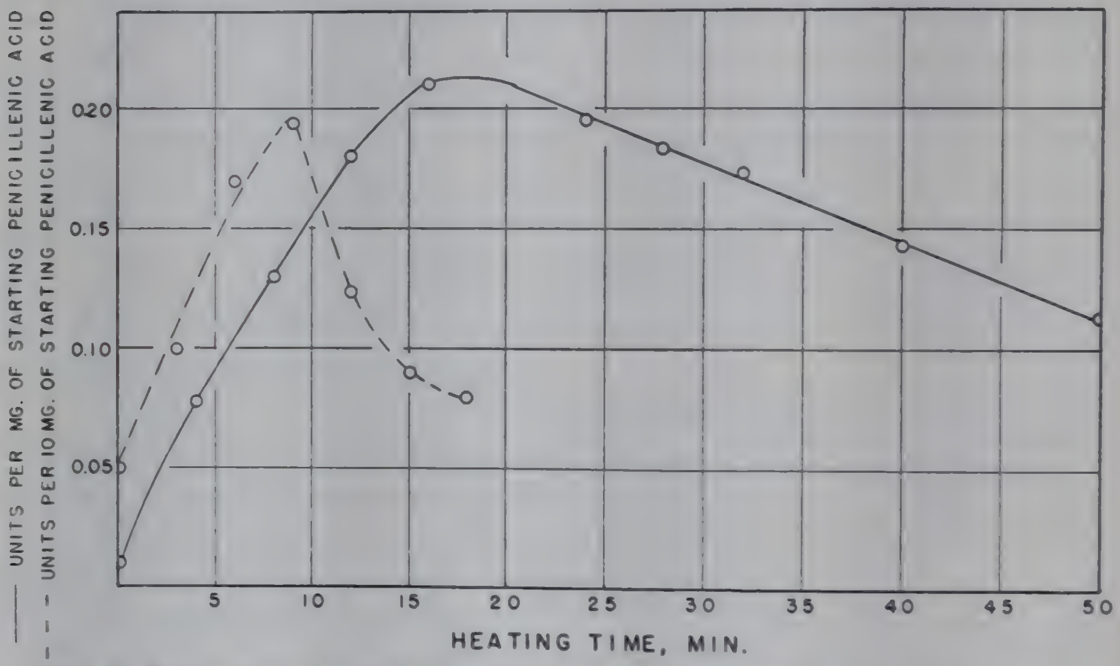


FIG. 2. Production of antibiotic activity on heating penicillenic acids in pyridine containing pyridinium chloride: penicillenic acid produced by the condensation of DL-β-methylcysteine, Isomer A, with 2-benzyl-4-methoxymethylene-5(4)-oxazolone (solid line); D-styrylpenicillenic acid (broken line).

Antibiotic Activity of Crude Synthetic Sodium Penicillins; Quantitative—The relative activities of the sodium salts of the crude synthetic penicillins were determined quantitatively against three microorganisms, *Staphylococcus aureus* H, *Bacillus subtilis* (ATCC 6051) and *Vibrio metchnikovii* (ATCC 7708). The activities were determined by an agar diffusion method with filter paper disks (Schleicher and Schüll, No. 740-E) (14, 15). Crystalline sodium benzylpenicillin was used as a standard in measuring the activities of each preparation against each organism. The assays involving the use of *Bacillus subtilis* and *Staphylococcus aureus* were essentially similar to those described by Foster and Woodruff (16) and Schmidt and Moyer (17) for these two organisms, with the exception that filter paper disks were used instead of cylinders.

The method developed for use with *Vibrio metchnikovii* was similar to that employing *Staphylococcus aureus* (17). In this assay, brain-heart infusion agar was used. First a layer of unseeded agar (12 cc.) was poured into sterile Petri dishes (100 mm. in diameter) with unglazed porcelain covers (Coors). After the agar had hardened, a layer of seeded agar (4 cc.) was added to each plate. Care was exercised to prevent the temperature of the agar seeded with *V. metchnikovii* from exceeding 45°. In order to prepare the inoculum, a slant of *V. metchnikovii* on infusion agar contained in a 15 × 150 mm. test-tube was incubated at 37° for 18 hours. The cells were washed from the slant with 5 cc. of sterile saline solution and 0.1 cc. of this suspension was used for every 4 cc. of agar. After the standard and unknown solutions had been added, the plates were incubated overnight at 37° and the diameters of the zones of inhibition of growth were measured. Since *V. metchnikovii* is less sensitive than *Staphylococcus aureus* to benzylpenicillin, the standard curve was determined by points ranging from 1 to 8 units of benzylpenicillin per cc. As the crude mixtures containing synthetic penicillin appeared to react gradually with water liberating an acidic group, it was necessary to use a strong buffer to prevent a drop in pH in solutions containing high concentrations of the test compounds. Therefore 1 M phosphate buffer at pH 7.0 was used to dissolve the standard sodium benzylpenicillin and the unknown samples. The results obtained on the assay of the crude mixtures containing synthetic penicillins are shown in Table I.

Qualitative—A study was made of the effect of the crude mixtures containing synthetic penicillins on the growth of seven organisms that were resistant to the action of benzylpenicillin. The organisms used were as follows: *Aerobacter aerogenes* (ATCC 8308), *Klebsiella pneumoniae* (ATCC 9997), *Mycobacterium smegmatis* (ATCC 101), *Mycobacterium tuberculosis* (ATCC 607), *Escherichia coli*, *Proteus vulgaris* OX-19, and *Pseudomonas aeruginosa*. The last three organisms were obtained from Professor James M. Neill.

Known amounts of the various synthetic reaction mixtures were placed in sterile Petri dishes. The crude sodium salt was dissolved in 1 cc. of 1 M phosphate buffer solution at pH 7.0, 9 cc. of brain-heart infusion agar (1.8 per cent) at 45° were added, and the resulting solution was mixed thoroughly. Each synthetic reaction mixture was tested at levels of 5, 10, and 20 mg. per cc. of agar. After the agar had hardened, each plate was streaked in a single line with a loopful of each of the seven organisms grown in broth. The plates were then incubated at 37° for 16 to 18 hours and read by macroscopic examination to see whether or not growth had occurred. The following control plates were prepared and used with each experiment: plates containing 1 cc. of buffer solution but no added penicillin, plates to which 1 cc. of buffer solutions (1 M phosphate at pH 7.0) of various concentrations of crystalline sodium benzylpenicillin was added so that the final plates contained 1 to 100 units (0.6 to 60 γ) of sodium benzylpenicillin per cc. of agar, and plates containing 5, 10, and 20 mg. (or about 5, 10, and 20 units) of crude synthetic sodium benzylpenicillin per cc. of agar. The broth cultures of the organisms used to streak the plates were prepared as follows: *Proteus vulgaris* was grown for 2 days in brain-heart infusion broth, the two *Mycobacteria* were grown for 2 days in Dubos broth (18), and all the other organisms were grown for 1 day in nutrient broth.

The results of the above experiments indicated that the growth of the organisms, with the possible exception of the *Mycobacteria* and the *Proteus*, was not inhibited by any of the synthetic penicillins when the crude reaction mixtures were tested at levels up to 20 mg. per cc. of agar. Although the growth of *Mycobacteria* and *Proteus* was not inhibited by 100 units (60 γ) of crystalline sodium benzylpenicillin, their growth was inhibited by the plates containing 20 mg. of a crude synthetic benzylpenicillin preparation per cc. of agar and by similar quantities of crude preparations of the other synthetic penicillins. Since the crude synthetic benzylpenicillin preparation contained only about 1 unit per mg., it was obvious that the growth of the organisms was being inhibited by substances in the reaction mixture other than penicillin. Further evidence on this point was obtained when the synthetic penicillins were assayed against *Mycobacterium tuberculosis* by an agar diffusion method with filter paper disks. The assay was similar to that described above for *Vibrio metchnikovii* with the exception that *Mycobacterium tuberculosis* was used as the test organism, and the Dubos medium (18) containing 1.5 per cent agar was used in place of the brain-heart infusion agar. In this assay, none of the crude synthetic penicillins including the crude sodium benzylpenicillin produced a zone of inhibition when tested at a concentration of 20 mg. per cc. Under the same conditions, 10 units of streptomycin per cc. produced a zone of inhibition measuring 21 mm. in diameter.

Fractional Crystallization of D-*Styrylpenicillenic Acid and Conversion of Fractions to Crude* D-*Styrylpenicillin*—600 mg. of D-styrylpenicillenic acid (Fraction A) were dissolved in 60 cc. of hot chloroform and 60 cc. of hot hexane were added. After the solution had cooled, 220 mg. of crystalline D-styrylpenicillenic acid (Fraction B) separated. Concentration of the mother liquor to dryness gave 237 mg. of residue (Fraction C). 180 mg. of Fraction B were recrystallized from 25 cc. of chloroform plus 21 cc. of hexane. The recrystallized product (Fraction D) weighed 55 mg. Concentration of the mother liquor to dryness gave 115 mg. of residue (Fraction E). Aliquots were removed from each fraction for the determination of melting point and molar absorption at 375 mμ in 95 per cent ethanol, and for conversion to crude D-styrylpenicillin (Table II).

In order to convert D-styrylpenicillenic acid to D-styrylpenicillin, the fractions of the D-styrylpenicillenic acid were dissolved so as to give solutions with a concentration of 10 mg. per cc. in pyridine containing 6.5 mg. of pyridinium chloride per cc. The resulting solutions were all placed at the same time in an oil bath at 130°. After 15 minutes the solutions were removed from the bath, 0.10 cc. of triethylamine was added per cc. of solution, and the resulting solutions were cooled in an ice bath. The solvents were removed *in vacuo*, and the residues were dissolved in various amounts of 1 per cent phosphate buffer at pH 7.0 and assayed against *Staphylococcus aureus* with crystalline sodium benzylpenicillin as the standard. For each fraction, the ratio of antibiotic activity produced to molar absorption at 375 mμ was calculated (Table II). These ratios remained quite constant, ranging from 4.48×10^{-7} to 4.95×10^{-7}.

TABLE II

Properties of Fractions from Recrystallization of D-Styrylpenicillenic Acid

Fraction	Melting point (decomposition)	E_M at 375 mμ (*a*)	Antibiotic activity produced (*b*)	$\frac{(b)}{(a)} \times 10^7$
	°C.		*unit per mg. starting fraction*	
A	127–130	37,400	0.0175	4.68
B	146–148	42,300	0.0190	4.50
C	112–121	25,250	0.0125	4.95
D	146–148	42,300	0.0195	4.61
E	122–127	35,700	0.0160	4.48

The authors wish to thank Dr. Herbert E. Carter for a generous supply of Isomers A and B of DL-β-methylcysteine. They also wish to thank Miss Josephine E. Tietzman for carrying out the microanalyses and Miss Mary R. Lloyd and Miss Helen E. Heath for aid with the penicillin assays.

SUMMARY

The synthesis in minute yields and the preparation in the form of crude sodium salts of several penicillins have been described. Some of the penicillins were analogues of benzylpenicillin in which the D-penicillamine moi-

ety of benzylpenicillin was replaced by either DL-β-methylcysteine, Isomer A; DL-β-methylcysteine, Isomer B; DL-β,β-diethylcysteine; or DL-β-ethyl-β-methylcysteine. In addition crude sodium D-phenylpenicillin and D-styrylpenicillin were synthesized.

In the first step of the two-step reaction used in the synthesis of these penicillins, appropriate oxazolones were condensed with various α-amino-β-mercapto acids to yield penicillenic acids. In most cases, the penicillenic acids were isolated as amorphous solids which were characterized by their ultraviolet absorption spectra. In the case of D-styrylpenicillenic acid, the intermediate penicillenic acid was obtained in crystalline form. In the second step of the synthesis, the penicillenic acids were converted in small yield to the corresponding penicillins. It was found that the optimum conditions for this rearrangement were dependent among other things upon the type of penicillenic acid involved.

The relative antibiotic activities of the crude preparations of the synthetic penicillins were determined on three microorganisms: *Staphylococcus aureus* H, *Bacillus subtilis*, and *Vibrio metchnikovii*. In qualitative tests it was found that the crude synthetic penicillins did not possess marked antibacterial properties against *Aerobacter aerogenes*, *Klebsiella pneumoniae*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Escherichia coli*, *Proteus vulgaris* OX-19, or *Pseudomonas aeruginosa*. These organisms also showed a high degree of resistance to the action of crystalline sodium benzylpenicillin.

Studies on crystalline D-styrylpenicillenic acid have added to the evidence that penicillenic acids are intermediates in the synthesis of penicillins in the reaction of appropriate oxazolones with α-amino-β-mercapto acids.

BIBLIOGRAPHY

1. du Vigneaud, V., Carpenter, F. H., Holley, R. W., Livermore, A. H., and Rachele, J. R., *Science*, **104**, 431 (1946).
2. Clarke, H. T., Johnson, J. R., and Robinson, R., The chemistry of penicillin, Princeton, in press.
3. du Vigneaud, V., Carpenter, F. H., Holley, R. W., Livermore, A. H., and Rachele, J. R., *cf.* (2) chapter 28.
4. du Vigneaud, V., Wood, J. L., and Wright, M. E., *cf.* (2) chapter 28.
5. Livermore, A. H., Carpenter, F. H., Holley, R. W., and du Vigneaud, V., *J. Biol. Chem.*, **175**, 721 (1948).
6. Carpenter, F. H., Turner, R. A., and du Vigneaud, V., *J. Biol. Chem.*, **176**, 893 (1948).
7. Committee on Medical Research, Office of Scientific Research and Development, Washington, and Medical Research Council, London, *Science*, **102**, 627 (1945).
8. Editorial Board of the monograph on the chemistry of penicillin, *Science*, **106**, 503 (1947).
9. Behrens, O. K., *cf.* (2) chapter 19.
10. Cornforth, J. W., *cf.* (2) chapter 21.

11. Carter, H. E., Stevens, C. M., and Ney, L. F., *J. Biol. Chem.*, **139**, 247 (1941).
12. du Vigneaud, V., Stacy, G. W., and Todd, D., *J. Biol. Chem.*, **176**, 907 (1948).
13. Crooks, H. M., *cf.* (2) chapter 16.
14. Vincent, J. G., and Vincent, H. W., *Proc. Soc. Exp. Biol. and Med.*, **55**, 162 (1944).
15. Sherwood, M. B., Falco, E. A., and de Beer, E. J., *Science*, **99**, 247 (1944).
16. Foster, J. W., and Woodruff, H. B., *J. Bact.*, **47**, 43 (1944).
17. Schmidt, W. H., and Moyer, A. J., *J. Bact.*, **47**, 199 (1944).
18. Pierce, C., Dubos, R. J., and Middlebrook, G., *J. Exp. Med.*, **86**, 159 (1947).

STUDY OF CARBON DIOXIDE FIXATION IN THE SYNTHESIS OF CITRULLINE*

By SANTIAGO GRISOLIA† and PHILIP P. COHEN

(*From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison*)

(Received for publication, July 29, 1948)

The ornithine → citrulline → arginine cycle of urea synthesis originally proposed by Krebs and Henseleit (1) has had increasing experimental support (2–10). The position of citrulline in the cycle, however, has been questioned (11). With the advent of the successful separation of the two over-all enzymatic steps, ornithine → citrulline and citrulline → arginine (10), from the intact cellular system the position of citrulline appeared to be well established. However, it seemed desirable to determine the intermediary rôle of citrulline by the use of carbon dioxide containing C^{14}. In the present paper it is demonstrated that the incorporation of C^{14} into the carbonyl group of citrulline and urea is of such a magnitude that citrulline must be considered as an obligatory intermediate in the urea synthesis cycle.

Procedures

Tissue Preparations—The enzyme preparations used in this study were the KCl-washed rat liver residue for the step ornithine → citrulline (10) and whole liver homogenate for the step citrulline → urea, both previously described by Cohen and Hayano (9).

Substrates—L-Ornithine and L-citrulline were prepared from L-arginine according to the method of Hunter (12) and Gornall and Hunter (13). Adenosine triphosphate (ATP) was prepared from rabbit muscle (14).

Analytical—Citrulline was estimated by the colorimetric method of Archibald (15). Urea was determined either by the manometric method of Krebs and Henseleit (1) or the colorimetric method of Archibald with isonitrosopropiophenone (16). The measurement of radioactivity was carried out according to Reid (17) with a thin mica window counter. Preparations of C^{14}-containing samples for counting were collected and dried on thin aluminum cups of known area. For orientation purposes some preparations were counted directly as dry films after adsorption of small samples on a layer of lens paper filling the bottom of aluminum cups. With care, reproducible results are obtained with this technique.

---

* Aided in part by a grant from the Wisconsin Alumni Research Foundation.

† Del Amo Foundation Fellow.

Results

Preliminary small scale experiments were carried out in which total $C^{14}O_2$ fixation was estimated for the step ornithine → citrulline (Table I). It will be noted that in all three experiments the elimination of either glutamic acid or ornithine results in a marked decrease in CO_2 fixation of the order of one-tenth to one-twelfth that of the complete system.

A large scale experiment was carried out under the conditions described in Table II. The reaction was stopped by the addition of 5.0 ml. of 1 N HCl and deproteinized by heating. The CO_2 liberated on the addition of acid was collected in alkali. Aliquots of the alkali samples containing the $C^{14}O_2$ were analyzed for total carbonate content by the standard Warburg manometric technique. The residual $C^{14}O_2$ was then precipitated as $BaCO_3$ for determination of radioactivity. Any residual $C^{14}O_2$ present in the reaction mixture was washed out by flushing with non-isotopic CO_2, which was then removed by prolonged flushing with CO_2-free air. The precipitated protein was centrifuged off, the supernatant filtered through retentive paper, and aliquots of the filtrate analyzed for citrulline and urea. 48 micromoles of citrulline were found and no urea was detected. Evapo-

TABLE I

Preliminary Experiments for Estimation of $C^{14}O_2$ Fixation

Experiment	Incubation mixture	$C^{14}O_2$ fixed in protein-free medium*
A	Complete system	100
"	Without ornithine	10
"	" glutamic acid	3
"	" " " and NH_3	9
B	Complete system	100
"	Without ornithine	8
"	" " fumaric acid replacing glutamic acid	10
C	Complete system	100
"	Without ornithine	12
"	" glutamic acid	10

Final substrate concentrations were as follows: 3.8×10^{-2} M L-glutamate, 2.5×10^{-3} M L-ornithine, 5×10^{-3} M NH_4Cl, 1.25×10^{-2} M phosphate buffer, pH 7.15, 3.3×10^{-3} M $MgSO_4$, 1.5×10^{-3} M ATP, 6×10^{-3} M $NaHC^{14}O_3$, and potassium ions to bring the medium to isotonicity and a total volume of 4.0 ml. Each cup contained 3.5 mg. of washed residue N. Incubation time 40 minutes at 38°.

* The radioactivity of the protein-free medium is expressed as relative values. 100 is equivalent to a total fixation of about 25 to 30 per cent of the C^{14} added.

ration of an aliquot to a dry film and assay for radioactivity indicated that a high CO_2 fixation had occurred.[1]

The reaction mixture after incubation and deproteinization was divided into three fractions which were diluted with non-isotopic L-citrulline. The dilutions were 5, 50, and 150 times the original citrulline present. These samples were now treated with $Ba(OH)_2$ and ethyl alcohol, according to Jones and Moeller (18), in order to remove dibasic amino acids. The precipitates were collected and aliquots assayed for radioactivity. All showed some activity due in part to the presence of some adsorbed citrulline on the crude barium salts. The precipitates were then resuspended in H_2SO_4. The $BaSO_4$ which was separated by centrifugation contained no appreciable radioactivity. Treatment of the supernatant with non-isotopic L-citrulline and further precipitation with $Ba(OH)_2$ and alcohol resulted in a drop in radioactivity of the precipitate. As the activity of these precipitates was very low, isolation and identification of this fraction were not undertaken in the present studies. For precipitation of the citrulline the method of Vickery and Gordon (19) employed for other amino acids was used. The supernatant from the first $Ba(OH)_2$ treatment was freed of alcohol by evaporation and brought to pH 7.0 by the addition of H_2SO_4. After removal of $BaSO_4$, the solution was treated with $HgCl_2$ to twice the molarity of the citrulline present, and then $Ba(OH)_2$ was added to raise the pH to 9.3. The citrulline-Hg-Ba complex was separated by centrifugation, washed several times with water, alcohol, and ether, and then assayed for

TABLE II

Radioactivity of Carbon Dioxide, Citrulline, and Urea

Compound	Specific activity, counts per min. per micromole compound
Carbon dioxide	420
Citrulline	413
Urea	410

The reaction mixture for citrulline synthesis contained the following components: 3.8×10^{-2} M L-glutamate, 3.3×10^{-3} M L-ornithine, 6.6×10^{-3} M NH_4Cl, 2×10^{-3} M ATP, 1×10^{-2} M phosphate buffer, pH 7.15, 3.3×10^{-3} M $MgSO_4$, 9×10^{-3} M $NaHC^{14}O_3$, and KCl ions to bring the medium to isotonicity (activity of C^{14} measured in a Geiger-Müller counter, 36,000 counts per minute per mg. of carbon). Washed residue 28 mg. of N. The total volume of the reaction mixture was 30 ml. The mixture was incubated at 38° for 40 minutes in a closed vessel and in the presence of air.

---

[1] It has been observed that the metabolic CO_2 production under these conditions will account for a 5 per cent maximum dilution of the $C^{14}O_2$ added.

radioactivity. The analytical values obtained with all three dilutions were consistent within 5 per cent. The samples were then taken up in dilute H_2SO_4, decomposed with H_2S, centrifuged, washed, and the supernatant and washings filtered through retentive paper. The filtrate was aerated and analyzed for citrulline. The samples were concentrated *in vacuo* to a small volume, and aliquots were converted enzymatically to urea according to Cohen and Hayano (10) except that aspartic acid was used instead of glutamic acid. Urea was separated either as xanthydrol urea according to the method of Allen and Luck (20) or decomposed with urease and the CO_2 precipitated as $BaCO_3$ in the usual way.

As can be seen from Table II the specific activity per micromole of urea and citrulline is practically the same as that of the bicarbonate of the medium.

Another procedure[2] employed in a large scale experiment for estimating the incorporation of $C^{14}O_2$ into citrulline involved the quantitative adsorption of citrulline from the deproteinized reaction mixture by Zeo-Karb according to the method reported by Archibald (15). Measurements of citrulline and radioactivity indicated quantitative adsorption of both by Zeo-Karb. Approximately 80 per cent of the citrulline was eluted from the Zeo-Karb by treatment with 30 per cent H_2SO_4. Analysis of the eluate after removal of sulfate ions with $Ba(OH)_2$ revealed the same ratio of radioactivity to citrulline content as before adsorption. The eluted citrulline solution was then heated at 105° for 1 hour with concentrated sodium hydroxide to decompose the terminal ureide group to carbon dioxide which was collected as $BaCO_3$. Measurement of radioactivity of this carbon dioxide showed a specific activity per mg. of carbon which was 98 per cent that of the original bicarbonate in the medium. Of interest was the finding that washing Zeo-Karb with 30 per cent H_2SO_4 gives rise to a substance in the washings which when heated with alkali reacts with diacetyl monoxime, thus interfering slightly with the determination of citrulline.

DISCUSSION

The synthesis of citrulline and urea with practically the same specific activity as that of the $C^{14}O_2$ present originally in the medium strongly supports the position of citrulline as an obligatory intermediate in the urea cycle. The relatively low rate of $C^{14}O_2$ fixation in the absence of ornithine or glutamic acid indicates that in this system citrulline synthesis is by far the most active CO_2 fixation reaction.

Attempts to demonstrate the formation of an intermediate carbamylglutamic acid derivative (21) in this study were unsuccessful owing chiefly to the instability and rapid conversion of this compound. Experiments in this direction are now in progress.

---

[2] This part of the study was carried out with the collaboration of Dr. M. Hayano.

The authors are indebted to Dr. Robert H. Burris, Department of Biochemistry, for samples of $C^{14}O_2$ and for facilities for estimation of radioactivity.

SUMMARY

1. The synthesis of citrulline from ornithine in the presence of $C^{14}O_2$ has been studied in washed rat liver residue. The specific activity of the isolated citrulline is of the same order of magnitude as that of the $C^{14}O_2$ added.

2. The conversion of citrulline containing C^{14} in the carbonyl position to urea by liver homogenates results in the formation of urea with the same specific activity as that of the added citrulline.

3. The significance of these findings on the intermediary rôle of citrulline in the Krebs-Henseleit cycle is discussed.

BIBLIOGRAPHY

1. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33 (1932).
2. Krebs, H. A., *Z. physiol. Chem.*, **217**, 191 (1933).
3. Krebs, H. A., *Biochem. J.*, **36**, 758 (1942).
4. Gornall, A. G., and Hunter, A., *J. Biol. Chem.*, **147**, 593 (1943).
5. Evans, E. A., Jr., and Slotin, L., *J. Biol. Chem.*, **136**, 805 (1940).
6. Rittenberg, D., and Waelsch, H., *J. Biol. Chem.*, **136**, 799 (1940).
7. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **153**, 401 (1944).
8. Rittenberg, D., Schoenheimer, R., and Keston, A. S., *J. Biol. Chem.*, **128**, 603 (1939).
9. Cohen, P. P., and Hayano, M., *J. Biol. Chem.*, **166**, 239 (1946).
10. Cohen, P. P., and Hayano, M., *J. Biol. Chem.*, **172**, 405 (1948).
11. Bach, J. S., *Biochem. J.*, **33**, 1833 (1939).
12. Hunter, A., *Biochem. J.*, **33**, 27 (1939).
13. Gornall, A. G., and Hunter, A., *Biochem. J.*, **33**, 170 (1939).
14. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis (1945).
15. Archibald, R. M., *J. Biol. Chem.*, **156**, 121 (1944).
16. Archibald, R. M., *J. Biol. Chem.*, **157**, 507 (1945).
17. Reid, A. F., Preparation and measurement of isotopic tracers, Ann Arbor (1946).
18. Jones, D. B., and Moeller, O., *J. Biol. Chem.*, **79**, 429 (1928).
19. Vickery, H. B., and Gordon, W. G., *J. Biol. Chem.*, **103**, 543 (1933).
20. Allen, F. W., and Luck, J. M., *J. Biol. Chem.*, **82**, 693 (1929).
21. Cohen, P. P., and Grisolia, S., *J. Biol. Chem.*, **174**, 389 (1948).

PURIFICATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE BY COUNTER-CURRENT DISTRIBUTION*

BY GEORGE H. HOGEBOOM† AND GUY T. BARRY‡

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

(Received for publication, July 19, 1948)

The isolation in the pure state of the coenzyme diphosphopyridine nucleotide (DPN) has constituted a difficult problem in chemical fractionation for a number of years. At the present time, it is possible by means of relatively simple procedures to obtain crude preparations containing 40 to 60 per cent DPN from yeast in good yield (1–3). Further purification of these crude preparations (4–7) usually involves precipitation of DPN with cuprous chloride, subsequent removal of acid impurities as insoluble salts of heavy metals, adsorption of DPN on columns of Al_2O_3, and finally fractional precipitation with alcohol. The procedure is tedious and is not easily reproducible with respect to the purity or yield of the final product.

The formidable nature of this method of purification has, in fact, led to the wide-spread use of crude DPN in enzyme studies and related investigations. Although in most instances the known specificity of the DPN-linked dehydrogenases leaves little doubt that DPN itself takes part in the enzyme reactions, the question continually arises as to whether the impurities, of which the chemical nature is largely unknown, include interfering compounds. It is obvious that a reproducible and simple method for obtaining pure DPN in good yield would be desirable.

In the present report a new method, based on the counter-current distribution principle developed by Craig (8), is described for the fractionation of crude preparations of DPN (purity approximately 60 per cent). Recent applications of this technique of fractionation to other complex mixtures (9–12) have amply demonstrated that it is a powerful tool in the separation and characterization of organic compounds. By utilizing a two-phase system consisting principally of phenol and water, it has been possible with relatively few transfers to isolate DPN of high purity (at least 96 to 98 per cent pure) in yields of 70 to 80 per cent.

---

* Aided in part by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

† Present address, National Cancer Institute, National Institute of Health, Bethesda 14, Maryland.

‡ Fellow of the American Cancer Society.

EXPERIMENTAL

Since the procedure of counter-current distribution depends upon the use of a two-phase system, some difficulty would be anticipated in attempts to apply the method to compounds characterized by high solubility in water and low solubility in the common organic solvents. DPN is, of course, an excellent example of this type of compound. Even when distributed in systems that contained large amounts of water in the organic solvent phase, such as 1-butanol-water or 2-butanol-water, over 99 per cent of the DPN was found in the aqueous phase ($K < 0.01$). Phenol and certain of its derivatives (*e.g. m*-cresol), however, constituted a group of organic solvents allowing for more favorable conditions for the counter-current distribution of DPN. In the system water-phenol at 6°, for example, approximately 90 per cent of the DPN was found in the organic phase ($K = 0.12$). A preliminary counter-current distribution of fifteen transfers was made at 6° with 50 mg. of crude DPN with the system 2 volumes of water-1 volume of phenol. The results of this distribution showed that most of the impurities in the preparation possessed a much higher distribution coefficient than did DPN itself. Furthermore, DPN did not undergo an appreciable degree of transformation during the procedure. With these data at hand, it was possible to make a more detailed analysis of crude preparations of DPN by means of the counter-current distribution technique.

Materials and Methods—The crude DPN fractionated in the present experiments consisted of several different lots of cozymase obtained from the Schwarz Laboratories[1] and containing approximately 60 per cent DPN.

Reagent grade crystalline phenol (Merck) was redistilled under reduced pressure and stored in the liquid form by the addition of 10 per cent water. All other solvents used were also redistilled in glass.

The counter-current distribution machine available for the present experiments was equipped with a glass plate at each end,[2] making it possible to observe directly the separation of the liquid phases. This improvement was particularly useful in the purification of DPN because the water-phenol system separated rather slowly and had a tendency to emulsify in the presence of some of the impurities in cozymase. The settling of most systems is indicated simply by transmission of light through the tubes. In the case of water-phenol, however, the layers were frequently cloudy, and a determination of separation was made by tilting the machine to the horizontal position for observation of the solvent interface by reflected light.

The DPN content of the cozymase and purified preparations obtained by distribution was estimated according to the method of Warburg and Christian (13) as modified at the Schwarz Laboratories.[3] A 3 to 5 mg. sample (weighed to 0.01 mg.) was dissolved in 2.00 ml. of a freshly prepared solution containing 0.20 per cent $Na_2S_2O_4$ and 1.0 per cent $NaHCO_3$. The mixture was placed in a boiling water bath for exactly 1 minute, immediately chilled in an ice bath, and diluted to an appropriate volume with a buffer containing 1.0 per cent $NaHCO_3$ and 1.0 per cent Na_2CO_3. The solution was oxygenated for 5 minutes and its optical density measured at 340 mμ in the Beckman spectrophotometer. This procedure was found to be superior to the usual method of reducing DPN over a period of several hours at room temperature (13) in that it gave more reproducible extinction coefficients.

---

[1] The authors are indebted to the Schwarz Laboratories, Inc., for a generous supply of cozymase.

[2] Craig, L. C., and Post, O., unpublished work.

The extinction coefficient at 340 mμ afforded a precise measurement of the relative increase in purity and the yield of DPN obtained in the fractionation procedure. Although the extinction coefficient of pure DPN reduced in solution with hydrosulfite has not been definitely established, for purposes of convenience a value of 8.5 sq. cm. per mg., according to LePage (3), was employed to estimate the DPN content of the cozymase and the samples of purified DPN.

Analysis of Crude DPN by Means of Counter-Current Distribution—Fig. 1 shows the results of a twenty-four transfer distribution of 202 mg. of cozymase. The components of the system for distribution were equilibrated in the following proportions before the experiment: 200 ml. of water, 100 mg. of KCl, 90 ml. of phenol, and 10 ml. of ether. Each tube of the machine contained 12 ml. of the upper (aqueous) layer and 7.8 ml. of the lower (phenol-ether) layer. The pH of the aqueous layer was approximately 5. The distribution was carried out in a cold room at 6° in order to minimize hydrolysis of DPN.

Potassium chloride was added because it had been previously noted that the addition of a small quantity of a strong electrolyte to the water-phenol system prevented to a considerable extent the formation of emulsions. It was necessary to use an electrolyte which would not be an objectionable contaminant in preparations of purified DPN and which was soluble in ethanol, since the subsequent isolation of DPN involved precipitation from aqueous solution with ethanol. KCl satisfied these requirements reasonably well. Ether was added to raise the distribution coefficient of DPN in the water-phenol system. As can be seen in Fig. 1, the addition of ether gave a higher distribution coefficient ($K = 0.59$) which was more favorable for fractionation.

Upon completion of the distribution, the mixture in each tube of the machine was transferred to a glass-stoppered test-tube and extracted three times with 15 ml. of ether. The initial ether extraction resulted in the transfer to the aqueous phase of the material dissolved in the phenol and in the removal of the phenol from the system. Two additional extractions were carried out in order to insure complete removal of phenol. There remained twenty-five aqueous solutions containing the components of the cozymase and the KCl. These solutions were analyzed by determining their optical density at 260 mμ and the results plotted as shown in Fig. 1. The central band, which represented DPN, coincided very closely with the theoretical distribution of a single substance possessing a distribution coefficient of 0.59. There was, however, a slight deviation beginning at Tube

---

[3] Gutcho, S., and Stewart, E. D., unpublished work.

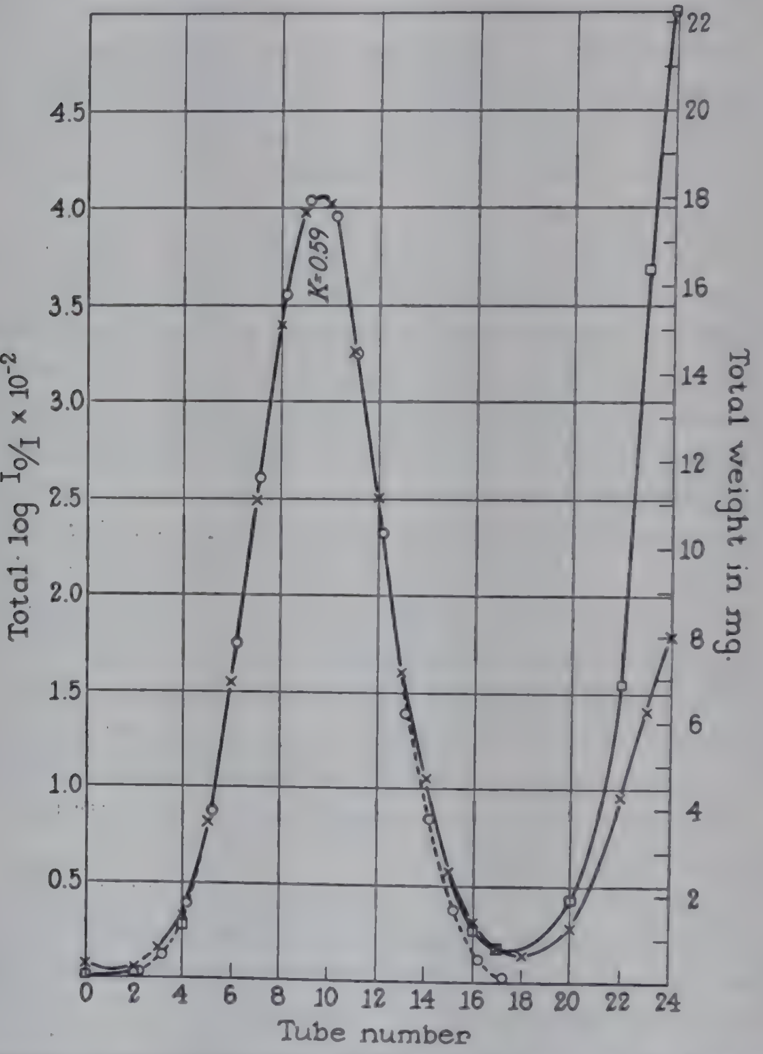


FIG. 1. Counter-current distribution of 202 mg. of cozymase in the system water-phenol-ether. ×, total optical density at 260 mμ; ○, theoretical distribution of a single substance possessing a distribution coefficient of 0.59; □, total weight in mg. after evaporation to dryness.

13 and extending to Tube 17, indicating the presence of another substance capable of absorbing light at 260 mμ. On the basis of absorption measurements, practically all of the remaining impurities in the cozymase were in Tubes 18 to 24. It should be mentioned that the impurities in Tubes 23 and 24 were troublesome during the distribution because they tended to cause emulsification of the system, making it necessary to wait 6 to 8 minutes between transfers.

Since by the analytical method only substances absorbing light at 260 mμ could be detected, additional data were necessary before the central band could be satisfactorily interpreted. Accordingly, a number of samples from tubes on both sides of the band were evaporated to dryness and the weight of the residue determined. After correction for the amount of KCl present, the weights were plotted in Fig. 1 on a scale approximating the weight of material in the tubes of the central band. The latter values were estimated from the extinction coefficient at 260 mμ of the DPN isolated from Tubes 6 to 12, inclusive. A consideration of the amount of material present in Tubes 2, 4, 16, and 17 led to the conclusion that the central band, on a weight basis, could have been no broader than the band determined by absorption measurements and shown in Fig. 1. This finding confirmed the results of a preliminary experiment involving fifteen transfers, in which it was found that the total weight of material present in each tube of the band representing DPN corresponded exactly to the optical density at 260 mμ. It was concluded, therefore, that Tubes 4 to 12 of the central band contained, except for KCl, either a single substance or a mixture of substances possessing identical distribution coefficients in the system.

Tubes 18 to 24 contained impurities amounting to 27 per cent of the weight of the original cozymase. The absorption curve of the material in these tubes showed a pronounced maximum at 260 mμ, typical of a compound or compounds containing adenine. On reduction with hydrosulfite, however, the absorption of these impurities did not increase at 340 mμ, a finding that showed the absence of compounds containing quaternary pyridinium nitrogen. The large deviation between the weights and the absorption measurements in Tubes 22 to 24 of Fig. 1 demonstrated the presence of impurities other than those absorbing at 260 mμ.

Although very little residue remained after evaporation to dryness of the aqueous solutions obtained from Tubes 0 and 2 (Fig. 1), it should be mentioned that a yellow precipitate formed at the interface on ether extraction of the contents of Tubes 0, 1, and 2. This precipitate, which represented an impurity possessing a low distribution coefficient, was lost during the ether extraction, and its weight could not be estimated.

Isolation of DPN—Immediately after completion of the absorption analysis, the aqueous solutions obtained from Tubes 6 to 12 were combined, filtered with suction through an inverted filter, frozen in a dry ice-acetone mixture, and lyophilized. The resulting solid material was dissolved in 4 ml. of water, and a white flocculent precipitate was obtained on addition of 40 ml. of cold absolute ethanol. The precipitate was recovered by centrifugation, washed with absolute ethanol and ether, and dried over P_2O_5 *in vacuo*. A white solid weighing 92 mg. was obtained. In an attempt to decrease the possibility of contamination with KCl, the nucleotide was redissolved in 3 ml. of water and reprecipitated with ethanol. The second precipitation presented some difficulties, however, because the compound formed an extremely fine precipitate consisting of particles of fairly uniform size (diameter about 0.4 μ) that did not coalesce over a period of 18 hours at 0°. When recovered by centrifuging at 2400 $\times g$ for 1 hour and dried over P_2O_5 *in vacuo*, the reprecipitated material weighed 82 mg. After reduction with hydrosulfite, its extinction coefficient at 340 mμ was 8.13 sq. cm. per mg., indicating a purity of 96 per cent. The starting material contained approximately 62 per cent DPN (E_{340} = 5.3 sq. cm. per mg.).

Fractionation of Cozymase in System, Water-Phenol-Chloroform—The experiment shown in Fig. 1 demonstrated that most of the impurities in the cozymase possessed a much higher distribution coefficient than did DPN in the system, water-phenol-ether, and could therefore be effectively removed by a few transfers. It was evident, however, that twenty-four transfers were required to eliminate impurities possessing a low distribution coefficient. That such impurities were likely to occur in crude preparations of DPN was apparent from the detection of solid material in Tubes 0, 1, and 2, which appeared on extraction with ether but was lost on removal of the ether. It was therefore desirable to devise a system in which the distribution coefficient of DPN was approximately 1. By this means it would be possible to separate with relatively few transfers impurities possessing both high and low distribution coefficients. The addition of more than 10 per cent ether to the phenol layer of the water-phenol system, in order to obtain a distribution coefficient higher than 0.59, was not feasible, however, because the density of the phenol-ether phase then approached too closely that of the aqueous phase, and the system did not separate readily. Preliminary experiments in which increasing amounts of chloroform were added to the water-phenol system showed that a distribution coefficient of approximately 1 for DPN could be obtained when the three components were used in the following proportions: 15 ml. of water, 7 ml. of phenol, and 3 ml. of chloroform. When KCl was added to the aqueous phase at a concentration of 0.10 mg. per ml. of water, the system separated very rapidly.

A twenty-four transfer distribution of 600 mg. of crude DPN was then carried out in the water-phenol-chloroform system, each tube of the machine containing 12 ml. of the aqueous layer and 7.8 ml. of the organic solvent layer. The two phases were obtained by equilibrating before the experiment the components of the system in the following proportions: 200 ml. of water, 20 mg. of KCl, 70 ml. of phenol, and 30 ml. of chloroform. The preparation of DPN available for this experiment had been partially purified by a previous fifteen transfer distribution in the system, water-phenol-ether. The extinction coefficient of the starting material at 340 mμ after reduction with hydrosulfite was 7.4 sq. cm. per mg. (indicated purity, 87 per cent). A considerably greater amount of starting material (600 mg.) than in the previous experiment (Fig. 1) was used in order to test the feasibility of a larger scale procedure for the isolation of DPN. The experiment was carried out at 6°.

After twenty-four transfers, the contents of each tube were withdrawn from the machine, the phenol and chloroform removed by three extractions with 15 ml. of ether, and the resulting twenty-five aqueous solutions analyzed by a determination of optical density at 260 mμ. The results of this analysis are given in Fig. 2. The main band (Tubes 7 to 20), which represented DPN, was not the symmetrical type usually obtained in the counter-current distribution of a single substance but showed a precipitous rise from Tubes 7 to 10 and a more gradual decline from Tubes 12 to 19. Previous experience has shown that this type of skewed curve does not indicate the presence of impurities but is the result of a non-linear partition isotherm; *i.e.*, a shift in distribution coefficient with concentration. In view of the symmetry of the main band in the experiment shown in Fig. 1, when a relatively small amount of starting material was used (202 mg.), it was likely that the asymmetry of the band in Fig. 2 resulted from the high initial DPN concentration. A method has not as yet been devised for the calculation of theoretical curves for substances with non-linear partition isotherms. On the other hand, it has been found that the total width of the base of a skewed curve obtained with a pure substance is very nearly the same as that of the symmetrical theoretical curve. On inspection of the two curves in Fig. 2, the only detectable impurity in the main band on the basis of absorption measurements at 260 mμ occurred in Tubes 16 to 20. This impurity corresponded to that in Tubes 13 to 17 of Fig. 1. A small amount of impurity absorbing at 260 mμ was present in Tubes 21 to 24 (Fig. 2).

In order to compare the purity of the DPN on both sides of the band shown in Fig. 2, the material in Tubes 8 to 12 and in Tubes 13 to 18 was pooled separately, frozen, lyophilized, redissolved in 5 ml. of water, and precipitated by the addition of 10 volumes of absolute alcohol. Each precipitate was washed with ether and dried in a vacuum desiccator over P_2O_5. 220 mg. of DPN were isolated from Tubes 8 to 12, the extinction coefficient at 340 mμ after reduction with hydrosulfite being 8.36 sq. cm. per mg. (indicated purity, 98 per cent). Tubes 13 to 18 yielded 209 mg. of DPN, of which the extinction coefficient at 340 mμ after reduction was 8.26 sq. cm. per mg. (indicated purity, 97 per cent). Both preparations were amorphous but perfectly white in color. The slightly lower extinction coefficient of the DPN from Tubes 13 to 18 was probably due to small amount of impurity in Tubes 16 to 18. Reprecipitation of the DPN from Tubes 13 to 18 did not produce any change in its extinction coefficient. On the basis of extinction coefficients at 340 mμ, a total of 80 per cent of the DPN in the starting material was recovered.

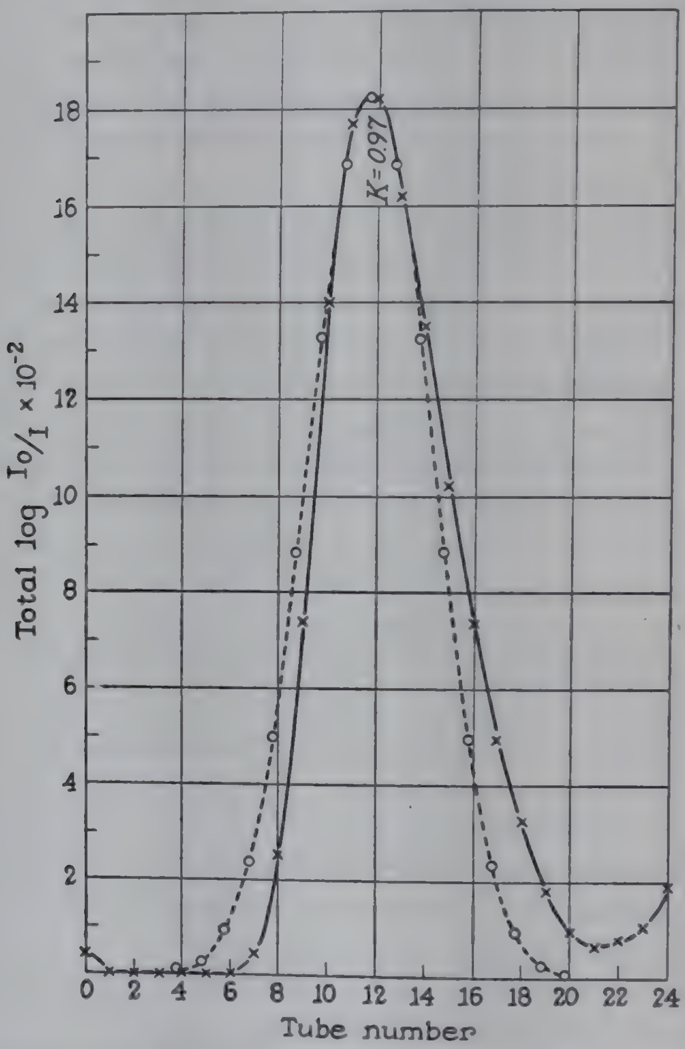


FIG. 2. Counter-current distribution of 600 mg. of partially purified DPN in the system water-phenol-chloroform. ×, total optical density at 260 mμ; ○, theoretical distribution of a single substance possessing a distribution coefficient of 0.97.

Procedure for Isolation of DPN on Large Scale—It was apparent from the data presented above that the use of an initial DPN concentration of much more than 3 per cent in the water-phenol-chloroform system would result in an excessive shift in the distribution coefficient. In larger scale procedures, it would therefore be necessary to increase the volume of the solvents above the capacity of the present counter-current distribution machine. The data also indicated, however, that the number of transfers required to effect satisfactory purification was sufficiently small to render the fractionation method feasible without resorting to the machine. Accordingly, the following experiment was carried out in order to test the practicability of a larger scale procedure for the isolation of DPN. Although only 1 gm. of cozymase was available for fractionation, it was evident that larger amounts could have been used if appropriate increases had been made in the volume of the solvents.

The first step in the purification was a four transfer distribution at 6° of 995 mg. of cozymase in the system, 12 ml. of water-12 ml. of phenol. The water phase contained 0.10 mg. of KCl per ml. Heavy glass-stoppered test-tubes were used for the distribution. After each equilibration, the tubes were centrifuged because of the tendency for the impurities possessing a high distribution coefficient to cause emulsification. The upper layers were transferred from one tube to the next by a vacuum-operated siphon. Tubes 0, 1, and 2 contained practically all the DPN because of its low distribution coefficient ($K = 0.12$) in the water-phenol system. Tubes 3 and 4 contained the bulk of the impurities of high distribution coefficient, corresponding to the material in Tubes 18 to 24 of the distribution shown in Fig. 1. The contents of each tube were then extracted three times with 15 ml. of ether. The resulting aqueous solutions obtained from Tubes 0, 1, and 2 were combined, frozen, and lyophilized. The residue was dissolved in 10 ml. of cold water, precipitated by the addition of 100 ml. of cold absolute alcohol, recovered by centrifugation, washed with ether, and dried *in vacuo* over P_2O_5. This preparation of partially purified DPN consisted of yellow resinous material and weighed 684 mg. After reduction with hydrosulfite its extinction coefficient at 340 mμ was 7.14 sq. cm. per mg. Since the extinction coefficient of the original cozymase was 5.25 sq. cm. per mg., the yield in terms of DPN recovered was 92 per cent. The purity of the DPN had been increased from 62 to 84 per cent.

The aqueous solutions from Tubes 3 and 4 were evaporated to dryness at 100°. Tube 3 contained 76 mg. and Tube 4, 144 mg. of impurity.

The second step in the isolation procedure consisted in distributing in the water-phenol-chloroform system the partially purified DPN obtained from Tubes 0, 1, and 2. This distribution was carried out under conditions similar to those of the experiment shown in Fig. 2. The number of transfers, however, was reduced to sixteen. 453 mg. of DPN were collected from Tubes 4 to 11, the extinction coefficient at 340 mμ being 8.25 sq. cm. per mg. after reduction with hydrosulfite. The indicated purity of the compound was therefore 97 per cent. The over-all yield of DPN obtained by the two distributions of 995 mg. of cozymase was 72 per cent.

Chemical Analysis of Purified DPN—Table I shows the results of elementary analysis of samples of DPN isolated from the distributions shown in Figs. 1 and 2. Both samples had been stored in a desiccator over P_2O_5 before analysis. Residual water was determined by heating the samples to constant weight at 100° *in vacuo*. In order to estimate the extent of contamination by KCl, chlorine analyses were carried out in addition to analyses for C, H, N, and P. It can be seen from the data in Table I that both samples gave low C, N, and P and high H values. Sample 2, which was purer than Sample 1 on the basis of extinction coefficients at 340 mμ (E = 8.36 *versus* 8.13 sq. cm. per mg.), gave C:N and N:P ratios in exact agreement with the theory, whereas the C:N ratio for Sample 1 was somewhat high. In general, the analytical values indicated that an appreciable amount of residual water was present in the samples even after they were dried at 100° *in vacuo*. Attempts to attain a completely anhydrous state were unsuccessful, however, because DPN decomposed when heated at temperatures higher than 100°.

TABLE I

*Analysis (Per Cent) of Samples of Purified DPN**

Sample	Residual water	C	H	N	P	Cl	C:N	N:P
Calculated for $C_{21}H_{27}N_7P_2O_{14}$ (mol. wt., 663.5)		38.01	4.10	14.78	9.35		2.572	1.58
Found, Sample 1†	5.46	37.75	4.44	14.38	9.08	Trace	2.62	1.58
" " 2‡	5.30	37.33	4.40	14.52	9.18	0.10	2.571	1.58

* Analyses were performed by Mr. D. Rigakos and Miss Theta Spoor of The Rockefeller Institute for Medical Research and by Dr. A. Elek, 4763 West Adams Boulevard, Los Angeles 16, California.

† DPN isolated from Tubes 6 to 12 of the distribution shown in Fig. 1.

‡ DPN isolated from Tubes 8 to 12 of the distribution shown in Fig. 2.

Activity of Purified DPN As Coenzyme—As a final check on the extent of purification of DPN, it was desirable to compare the activity of the purified DPN as a coenzyme with that of a crude cozymase preparation. The lactic acid dehydrogenase system of rat liver was found to offer a convenient method for estimating DPN by enzyme assay. This determination was based on the finding that the DPN-cytochrome *c* reductase activity of rat liver homogenates was much higher than the lactic acid dehydrogenase activity.[4] When sufficient cyanide was added to inhibit cytochrome oxidase, a determination of lactic acid dehydrogenase activity could be made by following spectrophotometrically at 550 mμ the rate of reduction of cytochrome *c*. Under the conditions noted below the reaction proceeded linearly with time for approximately 10 minutes, and the rate was proportional to the DPN concentration until relatively large amounts of DPN had been added.

---

[4] Hogeboom, G. H., unpublished work.

Fig. 3 shows a comparison of the coenzyme activity in the lactic acid dehydrogenase system of a cozymase preparation (E_{340} = 5.3 sq. cm. per mg., indicated purity 62 per cent) with that of purified DPN (E = 8.36 sq. cm. per mg., indicated purity 98 per cent). The reaction mixtures were made up by adding the following components in order: 0.20 ml. of 0.50 M K_2HPO_4-KH_2PO_4 buffer, pH 7.4; 0.20 ml. of 0.82 M nicotinamide; water to give a final volume of 3.00 ml.; 0.30 ml. of liver extract, diluted 1:50 with 0.88 M sucrose; 0.30 ml. of 10^{-3} M NaCN; 0.40 ml. of 2.2 × 10^{-4} M oxidized cytochrome *c*; varying amounts of DPN or cozymase dissolved in 0.05 M potassium phosphate buffer, pH 7.4; and 0.20 ml. of 0.11 M sodium lactate. The control reaction mixture contained all components except DPN.

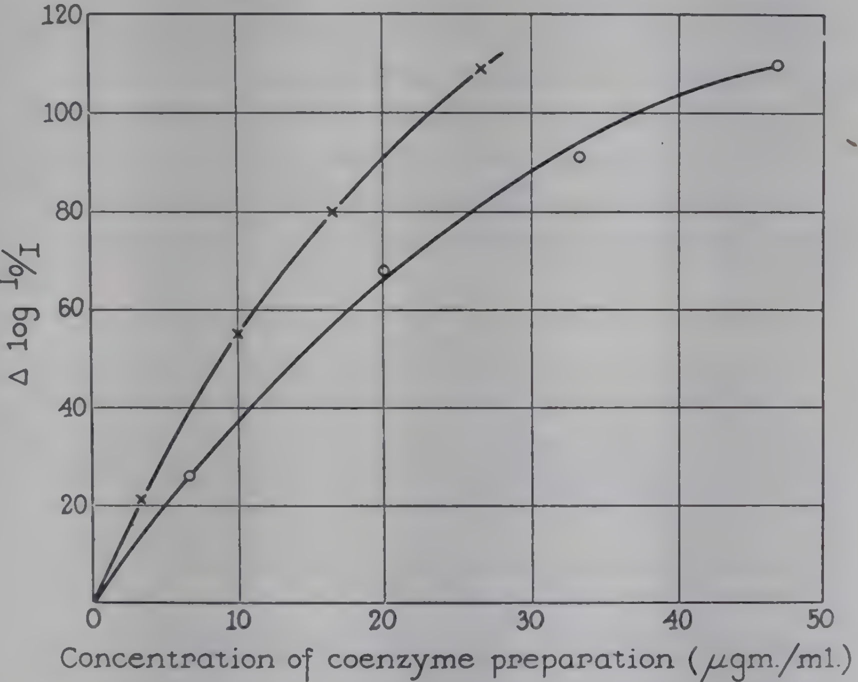


FIG. 3. Comparative activity of crude cozymase and purified DPN as hydrogen carriers in the lactic acid dehydrogenase system of rat liver. ×, increase in optical density at 550 mμ over a 5 minute period on the addition of increasing amounts of purified DPN; ○, increase in optical density at 550 mμ over a 5 minute period on the addition of increasing amounts of crude cozymase.

The liver extract was prepared as described previously (14) by homogenizing 1 gm. of rat liver in 9 ml. of 0.88 M sucrose, and centrifuging the homogenate for 20 minutes at 600 × g in order to remove residual intact cells and free nuclei. The cell-free supernatant was used as a source of lactic acid dehydrogenase and DPN-cytochrome *c* reductase. Cytochrome *c* was maintained in the oxidized form by the addition of HCl to a final concentration of 0.01 M. The experiment was carried out at 22°.

After the addition of sodium lactate, the reaction mixtures were quickly transferred to cuvettes and the increase in the optical density at 550 mμ followed at intervals of a minute in the Beckman spectrophotometer. In Fig. 3 the increase in absorption over a 5 minute interval is plotted against the concentration of DPN or cozymase. It can be seen that at each level of enzyme activity the approximate ratio of crude cozymase to purified DPN yielding an equivalent reaction rate was 1:0.63. The results of this experiment are in agreement with the DPN content of the two preparations, as calculated from the extinction coefficients at 340 mμ after reduction with hydrosulfite. The experiment also demonstrated that the DPN isolated by the counter-current distribution method is active as a coenzyme.

DISCUSSION

Purity of DPN Isolated by Counter-Current Distribution—On the basis of several criteria of homogeneity, it is probable that the DPN obtained by the counter-current distribution of crude preparations of cozymase contained, aside from residual moisture, no more than a few per cent of impurities. Although not an entirely reliable measure of purity, the extinction coefficient of the DPN at 340 mμ after reduction with hydrosulfite closely approached maximum values expected for pure DPN reduced under similar conditions. In this respect, Drabkin (15) has shown that the occurrence of either transformation or reoxidation during the reduction of DPN in solution results in significantly lower extinction coefficients than corresponding values obtained for reduced diphosphopyridine nucleotide ($DPNH_2$) isolated by Ohlmeyer's procedure (16). The extinction coefficient at 340 mμ for pure anhydrous $DPNH_2$ is 9.43 sq. cm. per mg. (16). On the basis of Drabkin's data, the value for pure anhydrous DPN, reduced in solution with hydrosulfite, was estimated to be approximately 8.8 sq. cm. per mg. The extinction coefficients of the preparations of DPN isolated by counter-current distribution, after correction for residual water content, were 8.63 to 8.83 sq. cm. per mg.

Elementary analysis of the purified DPN was rendered somewhat difficult by the fact that the compound was hygroscopic and contained residual moisture after having been stored over P_2O_5 for prolonged periods in a vacuum desiccator. A completely anhydrous state was apparently not attained even after heating the compound *in vacuo* at 100°. In general, it may be stated, however, that the analytical figures compared favorably with similar values reported in the literature for DPN considered to be pure (6, 7, 13, 17) and indicated that preparations containing at least 96 to 98 per cent DPN can be readily isolated by means of counter-current distribution.

Finally, it should be pointed out that the curves representing DPN (Figs. 1 and 2) obtained from the counter-current distribution of cozymase were in close agreement with the theoretical distribution of a single substance. It has been shown that agreement between experimental and calculated curves is an important criterion of homogeneity (9, 10, 12).

Remarks on Fractionation and Isolation Procedure—Counter-current distribution often, as in the case of the penicillins (9, 10), makes possible the separation and isolation of unstable compounds with a minimum of decomposition. In the present experiments with DPN, a substance that is easily hydrolyzed at both acid and alkaline pH, the temperature was maintained at 6° or below, and the pH of the aqueous phase of the system was approximately 5. In spite of the mildness of these conditions, however, a consideration of the curves shown in Figs. 1 and 2 brought up the possibility that a slight amount of transformation of DPN may actually have occurred. Thus the results of the distribution shown in Fig. 1 indicated that fragments of the DPN molecule containing adenine possessed a very high distribution coefficient and should have appeared almost exclusively in Tubes 23 and 24 of the experiment shown in Fig. 2. If a continuous, slow hydrolysis occurred during the distribution, however, these fragments would not be sharply localized in Tubes 23 and 24 but would extend from the right side of the DPN band to Tube 24. The fact that the total optical density of the material in Tubes 20 to 23 of Fig. 2 did not approach zero but remained almost constant could be accounted for on this basis.

Previous investigations (5) have shown that the weakest point of the DPN molecule occurs at the linkage between ribose and pyridine nitrogen and that the initial step in the hydrolysis of DPN yields free nicotinamide. In the method of isolation of DPN used in the present experiments, involving precipitation from aqueous solution with ethanol, free nicotinamide would be eliminated from the final preparation because of its solubility in ethanol. Hydrolytic products from the remainder of the molecule might be precipitated by ethanol and thus could occur as possible contaminants.

It should also be pointed out that heavy metals, which might possibly be present in small amounts in crude DPN, would remain in the aqueous phase of the water-phenol system and therefore would not contaminate DPN purified by counter-current distribution.

A comparison of the distribution coefficient of DPN and triphosphopyridine nucleotide (TPN) and an investigation of the behavior of the latter substance on counter-current distribution would be of considerable interest. TPN was not available in sufficient amounts, however, to permit such a study.

The authors are indebted to Dr. Lyman C. Craig for his interest and helpful suggestions.

SUMMARY

A method, based on counter-current distribution, is described for the fractionation of crude preparations of diphosphopyridine nucleotide (DPN). DPN of high purity (at least 96 to 98 per cent pure) was readily isolated in yields of 70 to 80 per cent by distribution in a two-phase system consisting principally of phenol and water. The procedure was demonstrated to be suitable for the isolation of pure DPN on a large scale.

BIBLIOGRAPHY

1. Williamson, S., and Green, D. E., *J. Biol. Chem.*, **135**, 345 (1940).
2. Jandorf, B. J., *J. Biol. Chem.*, **138**, 305 (1941).
3. LePage, G. A., *J. Biol. Chem.*, **168**, 623 (1947).
4. von Euler, H., Albers, H., and Schlenk, F., *Z. physiol. Chem.*, **240**, 113 (1936).
5. Schlenk, F., in A symposium on respiratory enzymes, Madison, 104 (1942).
6. von Euler, H., and Schlenk, F., *Z. physiol. Chem.*, **246**, 64 (1937).
7. von Euler, H., and Schlenk, F., *Svensk Kem. Tidskr.*, **48**, 135 (1936).
8. Craig, L. C., *J. Biol. Chem.*, **155**, 519 (1944).
9. Craig, L. C., Hogeboom, G. H. Carpenter, F. H., and du Vigneaud, V., *J. Biol. Chem.*, **168**, 665 (1947).
10. Barry, G. T., Sato, Y., and Craig, L. C., *J. Biol. Chem.*, **174**, 209 (1948).
11. Hogeboom, G. H., and Craig, L. C., *J. Biol. Chem.*, **162**, 363 (1946).
12. Sato, Y., Barry, G. T., and Craig, L. C., *J. Biol. Chem.*, **170**, 501 (1947).
13. Warburg, O., and Christian, W., *Biochem. Z.*, **287**, 291 (1936).
14. Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.*, **172**, 619 (1948).
15. Drabkin, D. L., *J. Biol. Chem.*, **157**, 563 (1945).
16. Ohlmeyer, P., *Biochem. Z.*, **297**, 66 (1938).
17. Schlenk, F., *J. Biol. Chem.*, **146**, 619 (1942).

THE ACTION OF ETHANOLAMINE, METHYLETHANOLAMINE, AND DIMETHYLETHANOLAMINE ON LIPIDE PHOSPHORYLATION*

By CAMILLO ARTOM and W. E. CORNATZER

(*From the Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, North Carolina*)

(Received for publication, June 2, 1948)

The administration of a single dose of choline stimulates the formation of phospholipides in the liver (2, 3) and in the small intestine (4) of rats on low protein diets. Like choline, ethanolamine is an essential constituent of phospholipides in tissues and one may suspect that it will exert a similar action. Moreover, choline can conceivably be formed by methylation of ethanolamine through the intermediate stages of methyl- and dimethylethanolamine. There is already rather extensive evidence for the occurrence of such a process in living organisms (5–11), although, in this respect, considerable species differences were noted.[1]

In the present study the action of ethanolamine, methyl-, and dimethylethanolamine on the formation of total phospholipides in the liver and small intestine has been investigated with the aid of radioactive phosphorus as an indicator.[2] The results have been compared with those of simultaneous experiments in which an equivalent amount of choline was given. Moreover, since apparently similar effects on the total phospholipides may actually be the result of quite different actions on the individual phospholipide fractions, in a few experiments the separation of the choline-containing from the non-choline-containing phospholipides of the liver has been attempted, and the determinations have been carried out on the separated fractions.

Since the completion of our investigation, of which the main findings have been reported in summarized form (1), a paper by Platt and Porter (14)

---

* Aided by a grant from the John and Mary R. Markle Foundation. Preliminary reports (1) have been presented before the North Carolina Academy of Science, Wake Forest, May, 1947, the Seventeenth International Physiological Congress, Oxford, July, 1947, and the American Society of Biological Chemists, Atlantic City, March, 1948.

[1] Unlike the rat (5) and a type III pneumococcus (6), the chick (12) and one mutant strain of *Neurospora crassa* (13, 8) seem to be unable to carry out the conversion of ethanolamine into methylethanolamine.

[2] The P^{32} was supplied by the Clinton Laboratories, Oak Ridge, Tennessee, on allocation from the United States Atomic Energy Commission. Part of the methyl- and dimethylethanolamine used in the experiments was graciously offered by the Carbide and Carbon Chemical Corporation, New York.

has appeared concerning the action of ethanolamine on the rate of formation of phospholipides in the liver. On this point, their and our results are substantially in agreement.

EXPERIMENTAL

Male albino rats (100 to 110 gm.) were maintained for 7 days on a low fat, low protein diet (Diet 26 (3)) in which a solution of pure B vitamins (15) was incorporated daily. Some of the animals received a single large dose of a commercial preparation of salad oil 18 hours before death. Three to seven animals were used in each experiment. One of them was used as a control and received 1 cc. of water by stomach tube. The others received one dose of the substances to be tested (1 cc. of a 0.2 M solution of the chlorides by stomach tube).[3] 5 minutes later the rats were injected intraperitoneally with a solution of Na_2HPO_4 (10 to 20 γ of P) containing 2 to 4 microcuries of radioactive phosphorus and after 6 hours they were killed by decapitation. The lipides were extracted from the liver and the small intestine and their radioactivity and phosphorus content determined. The analytical procedures and the method for calculating and expressing the results have been described (3).

For the separation of the choline-containing from the non-choline-containing phospholipides, the chloroform solution of the lipides extracted from one or more livers was used. After evaporation of the solvent, the residue was dissolved in methanol, and the solution filtered and treated according to Taurog *et al.* (16). The radioactivity, phosphorus, and often also choline (17) were determined in aliquots of the solution before and after adsorption on MgO.

Results

The data on the radioactivity and specific activity of the total phospholipides in the liver and small intestine are reported in Tables I and II. In order to evaluate the statistical significance of our results, the differences observed in each experiment between the rats receiving the substances and the control receiving water have been averaged and the t test of significance (18) applied to the averages.[4] The mean increase over the controls, the value of t, and the degree of probability (P) for a chance occurrence of the increase are indicated in Tables I and II.

---

[3] Ethanolamine (Eastman Kodak), methylethanolamine (Carbide and Carbon Chemical Corporation), dimethylethanolamine (Carbide and Carbon Chemical Corporation, Eastman Kodak). These substances were identified by determinations of specific gravity, boiling point, acid equivalent, ammonia evolved by alkaline periodate, and formol titration. The titer of the solutions of choline chloride (Merck and Company) was estimated by determinations of N and Cl.

[4] Substantially the same conclusions are reached if the statistical treatment is applied to the differences between the means of each experimental group.

TABLE I

*Total Radioactivity and Specific Activity of Phospholipides in Liver of Rats Receiving Water, Ethanolamine, Methylethanolamine, Dimethylethanolamine, and Choline**

Experiment No.	Total radioactivity					Specific activity				
	Water (controls)	Ethanolamine	Methyl-ethanolamine	Dimethyl-ethanolamine	Choline	Water (controls)	Ethanolamine	Methyl-ethanolamine	Dimethyl-ethanolamine	Choline
	r.r.u.	*r.r.u.*	*r.r.u.*	*r.r.u.*	*r.r.u.*					
1†	281	428			586	86	126			133
2†	426	465		581	485	86	128		123	96
3†	291	462		803	495	81	102		147	107
	385					85				
4†	408	559	592	734		106	132	110	159	
5	307	421			394	86	115			103
6	373	496		542	500	102	126		119	104
7	300	626		729		78	135		144	
8	352	429	636			93	150	179		
		558					152			
9	534		828	748		96		179	142	
			644					164		
10	402	500	535	579	475	96	118	122	163	115
		547			433		140			121
11	439	680			513	100	141			106
12	482		575	930		85		95	148	
Averages‡	383	514	635	705	485	91	130	141	143	110
	(398)	(532)	(643)	(705)	(463)	(92)	(134)	(148)	(143)	(110)
Mean increase over controls		147	183	316	119		37	46	53	19
t		6.89	4.68	7.23	4.19		8.89	3.01	9.33	4.39
P		<0.01	<0.01	<0.01	<0.01		<0.01	<0.05	<0.01	<0.01

* The radioactive values are expressed in relative radioactive units (r.r.u.), the dose of P^{32} injected into the rat being considered as 10^4 r.r.u. "Total radioactivity" is the number of r.r.u. in the lipides of the whole organ. "Specific activity" is the ratio of the total radioactivity to the mg. of lipide phosphorus in the organ of a 100 gm. rat. t is the test of significance as applied to the mean increase over the control of the same experiment. P is the degree of probability for a chance occurrence of this increase.

† These rats received 1.5 gm. of oil 18 hours before death (12 hours before receiving P^{32}).

‡ The figures in parentheses are the averages of the values obtained on the rats to which no oil was given.

It is apparent that, in the liver of the rats fed any of the substances tested, both the radioactivity and the specific activity are markedly increased and that all the increases have a considerable degree of statistical significance.

On the basis of the average values, the stimulating effect on the lipide phosphorylation in the liver is increasing in the order, choline, ethanolamine, methyl-, and dimethylethanolamine.

TABLE II

*Total Radioactivity and Specific Activity of Phospholipides in Small Intestine of Rats Receiving Water, Ethanolamine, Methylethanolamine, Dimethylethanolamine, and Choline**

Experiment No.	Total radioactivity					Specific activity				
	Water (controls)	Ethanolamine	Methyl-ethanolamine	Dimethyl-ethanolamine	Choline	Water (controls)	Ethanolamine	Methyl-ethanolamine	Dimethyl-ethanolamine	Choline
	r.r.u.	*r.r.u.*	*r.r.u.*	*r.r.u.*	*r.r.u.*					
1*	125	184			229	45	59			69
2*	213	206		224	236	61	74		81	64
3*	226	179		261	181	61	57		92	58
	194					59				
4*	199	156	189	239		61	66	59	77	
5	120	124			184	52	52			60
6	161	222		214	185	55	77		68	64
7	160	206		191		55	70		70	
8	109	116	128			41	48	58		
		168					60			
9	88		171	131		51		70	70	
			124					76		
10	167	172	211	215	160	51	60	71	74	67
		167			183		63			66
11	210	198			250	65	66			76
12	216		208	257		58		61	79	
Average*........	168 (154)	174 (171)	172 (168)	216 (201)	201 (192)	55 (53)	63 (62)	66 (67)	76 (72)	65 (66)
Mean increase over controls.		9	27	41	23		9	15	21	9
t...............		0.88	1.86	7.18	1.50		3.56	4.92	9.14	3.10
P...............		>0.05	>0.05	<0.01	>0.05		<0.01	<0.01	<0.01	<0.05

* See explanations for Table I.

Essentially similar effects have been observed in many, but not in all, experiments on the small intestine. Accordingly, the mean increases over the controls, especially for the total radioactivity, are often small and always less marked than in the liver. However, all the differences in the specific activity values are clearly significant. In this respect, it should be pointed out that our determinations on the small intestine have been made on the whole organ, *i.e.* on both mucosa and muscle. In the latter tissue, the rate of phospholipide turnover is lower and perhaps it is modified to a lesser degree (or not at all) by the substances given to the animals.

From the limited number of data available, it seems that the administration of oil 18 hours before death did not affect appreciably these results either in the liver or in the intestine.[5]

Table III shows the results of determinations made in order to test the reliability of the method for the separation of the choline- and non-choline-containing phospholipides in our lipide extracts. On the basis of the choline values, it appears that, after treatment with MgO, practically all of the choline-containing phospholipides (93 to 100 per cent) are left in the solution. However, the amount of phosphorus which escaped adsorption is

Table III

Total, Choline-Containing,† and Non-Choline-Containing‡ Phospholipides of Rat Liver before and after Adsorption on MgO*

Substance given	Sample	Before adsorption			After adsorption			Choline-containing phospholipide not adsorbed	Non-choline-containing phospholipide adsorbed
		Total phospholipide	Choline-containing phospholipide	Ratio, choline-containing to total	Total phospholipide	Choline-containing phospholipide	Ratio, choline-containing to total		
		mg.	*mg.*		*mg.*	*mg.*		*per cent*	*per cent*
Water	A	104	51	0.49	66	48	0.73	94	66
	B	83	44	0.53	58	41	0.71	93	56
	C	109	51	0.47	61	48	0.79	94	78
Ethanolamine	D	82	41	0.50	51	39	0.76	95	61
	E	105	56	0.53	66	53	0.80	95	72
	F	84	44	0.52	49	41	0.84	93	80
Methylethanolamine	G	92	53	0.58	59	52	0.88	98	82
	H	94	49	0.52	52	48	0.92	98	91
Dimethylethanolamine	I	102	59	0.58	65	57	0.88	97	81
	J	74	41	0.55	46	42	0.91	102	86
Choline	K	84	64	0.76	58	60	1.03	94	100
	L	86	62	0.72	61	61	1.00	98	100

* Total phospholipide = mg. of lipide P × 22.7.

† Choline-containing phospholipide = mg. of choline × 6.7.

‡ Non-choline-containing phospholipide = (mg. of total phospholipide) − (mg. of choline-containing phospholipide).

---

[5] However, in four out of five experiments the intestine of the control rats which had received oil exhibited values for the total radioactivity (and, to a lesser extent, also for the specific activity) which were higher than the general averages. Similar increases in the intestinal lipides of rats on Diet 26 after the administration of a large dose of oil 6 hours before death have been noted previously (3).

often greater than that which would have been expected from the choline figures, this excess representing from 9 to 44 per cent of the non-choline-containing phospholipides assumed to be present in the original extract. All of the non-choline-containing phospholipides seem to have been adsorbed only in the two experiments on the liver of rats which received choline. In these experiments the ratio of choline to phosphorus in the solution after adsorption approximates 1, as in the determinations of Taurog *et al.* (16). These findings suggest the possibility that, when choline or choline precursors are absent from the diet, increased amounts of substances containing phosphorus, but not choline, may be present in the lipide extracts of the liver and, like lecithin, remain in the methanol solution after treatment with MgO. This hypothesis is now being investigated. At present, it is apparent that, at least in most of our experiments, the significance of the results obtained by the procedure which we have used is open to question. With this reservation, in Table IV we have merely recorded the average data of these determinations and their standard deviations. After the administration of ethanolamine, methyl-, and dimethylethanolamine, the radioactivity values in both phospholipide fractions were consistently higher than the corresponding values in the controls receiving

TABLE IV

*Total Radioactivity and Specific Activity of Rat Liver Phospholipides in Fractions Not Adsorbed and Adsorbed by MgO**

Substance given	No. of analyses	Not adsorbed (choline-containing phospholipide)	Adsorbed (non-choline-containing phospholipide)	Per cent increase over controls		
				Choline-containing phospholipide (a)	Non-choline-containing phospholipide (b)	Ratio $\frac{(a)}{(b)}$
Water (controls)........	4	227 ± 8	141 ± 12			
		(91 ± 9)	(92 ± 29)			
Ethanolamine..........	5	310 ± 46	218 ± 21	37	54	0.7
		(131 ± 25)	(127 ± 12)	(47)	(36)	(1.3)
Methylethanolamine....	2	328 ± 14	205 ± 6	45	45	1.0
		(133 ± 3)	(128 ± 13)	(48)	(39)	(1.2)
Dimethylethanolamine..	3	416 ± 49	186 ± 17	83	32	2.6
		(156 ± 39)	(127 ± 6)	(77)	(38)	(2.0)
Choline................	3	318 ± 15	149 ± 19	40	5	8.0
		(118 ± 8)	(115 ± 9)	(31)	(25)	(1.2)

* The figures preceded by the ± sign indicate the standard deviations. The values in parentheses are those of the specific activity. For other explanations see Table I.

water. In the group receiving choline, the radioactivity was definitely elevated only in the choline-containing fraction.

When the changes in the radioactivity of the two fractions are compared with each other, the *absolute* increase was always higher in the choline-containing phospholipides. However, the *per cent* increase over the control value was greater in the non-choline-containing fraction after ethanolamine was given, and was approximately the same in the two fractions from the rats receiving methylethanolamine. On the other hand, the increase was proportionately higher in the non-choline-containing phospholipides after dimethylethanolamine was given, and, even more so, after choline administration.

As for the specific activities, in all experimental groups the values were higher than in the controls, in the choline-containing as well as in the non-choline-containing phospholipides. Except for the rats receiving dimethylethanolamine, the average figures were of the same order of magnitude in the two fractions. However, the individual data were quite irregular, and in the various experiments of the same group the specific activity was sometimes greater in the choline-containing and sometimes in the non-choline-containing fraction.

In view of these inconsistencies, it is not easy to state definitely whether or not any of the substances which we have tested stimulated preferentially the formation of lecithin or of cephalin. Indeed, under the conditions of our experiments, it seems that the evidence for a proportionately greater formation of one phospholipide fraction would be conclusive only if the per cent increase in the total radioactivity of this fraction were greater and if the specific activity were also consistently higher or, at least, not lower than that of the other fraction. However, it must be pointed out that, because of the likely presence in the lipide extracts of variable amounts of P-containing substances other than the typical lecithin and cephalin (see above), the computation of the specific activity in the separated fractions was probably subject to an error greater than that of the radioactive determinations. On the other hand, if lecithin and cephalin were synthesized in the liver from a common immediate precursor containing P (*e.g.* glycerophosphate or phosphatidic acid), the comparison between the radioactivity values would be a direct indication of the relative amounts of phospholipides newly formed in the two fractions.

DISCUSSION

Present findings show that in both liver and intestine of rats on a low fat, low protein diet the administration of a single dose of ethanolamine or of its products of partial methylation stimulates the formation of total phospholipides. The stimulation appears to be of the same order of magnitude or greater than that caused by the administration of an equivalent amount of choline.[6]

The effect of choline on lipide phosphorylation in the liver has been correlated with the action of this substance in preventing fatty liver (and hemorrhagic kidneys in weanling rats). Other lipotropic substances such as betaine (19) and methionine (20) also cause an increase in the formation of total phospholipides in the liver. Dimethylethanolamine, which in the present experiments stimulated markedly the lipide phosphorylation, was previously found to be lipotropic (7). On the other hand, ethanolamine is said to be ineffective (21, 22), although in very young rats the administration of this substance, alone or together with methionine, to a certain extent decreases the fat infiltration (23, 24) and prevents hemorrhagic kidneys (24). An even more definite lack of parallelism between stimulation of lipide phosphorylation in the liver and lipotropic activity has been noted for cysteine and cystine (20). In other words, from the present evidence, it appears that those substances which exert a marked lipotropic action also stimulate the formation of liver phospholipides, but that the reverse is not true. Apparently, the lipotropic action is more specific than the stimulation of lipide phosphorylation.

As mentioned above, the adequacy of the separation of the choline-containing and non-choline-containing phospholipides is open to question, and a further uncertainty in the interpretation of the data is introduced by the irregularity in the specific activity values as determined in the separated fractions. If it were assumed that in the conditions of our experiments the radioactivity data may be a reliable indication of the relative amounts of choline- and non-choline-containing phospholipides formed during the 6 hours of the experiments, it would appear that, after choline was given, the stimulation of lipide phosphorylation in the liver involves almost exclusively the choline-containing fraction, a finding which would be in line with previous results of Entenman *et al.* (25) on the liver of dogs receiving choline. On the other hand, after ethanolamine or its partially methylated derivatives were fed, the formation of both lecithin and cephalin is increased, but the extent of the relative increase in each fraction varies with the substance given.

As a tentative explanation of these findings, it may be pointed out that the protein level in our experimental diet was as low as 5 per cent, and that casein contains only minute amounts of glycine, a likely precursor for ethanolamine (5). In spite of the easiness with which glycine can be synthesized in the body, it is perhaps not unreasonable to assume that in our control rats the availability of ethanolamine, as well as that of choline, could represent a limiting factor for the synthesis of phospholipides. When large amounts of preformed ethanolamine were administered part of the substance given may have been utilized directly as a building stone for the formation of cephalin, and another part indirectly as a methyl acceptor for the synthesis of choline. One can thus understand that in these experiments an increased formation of both phospholipide fractions occurred, the synthesis of lecithin being then limited only by the amounts of available methyl groups. After methyl- or dimethylethanolamine was given, a more marked synthesis of lecithin became possible, since for each newly formed molecule of choline, only two, or one, additional methyl groups were required. When large amounts of choline are fed, it is probable that part of the choline reaching the liver is destroyed by the action of choline oxidase (26, 27) and thus is not used in the synthesis of phospholipides. On the other hand it is possible that dimethylethanolamine is introduced as such into the molecule of a phospholipide (or a phospholipide precursor) without previous methylation to choline, and by this process escapes the action of choline oxidase. This might perhaps explain the higher rate of lecithin formation after dimethylethanolamine is given than after choline administration.

---

[6] However, it should be pointed out that, according to our previous data (3), the choline effect on lipide phosphorylation in the liver and intestine of rats on Diet 26 is markedly enhanced by the simultaneous administration of fat.

As for the finding that not only ethanolamine but also its products of partial methylation caused an increase in the radioactivity of the non-choline-containing fraction, it is conceivable that in these experiments some ethanolamine originated from the compounds fed. Such a demethylation should probably occur through reactions other than those involved in transmethylation, since dimethylethanolamine apparently is not an effective methyl donor for the synthesis of methionine (7, 28). On the other hand, if the possibility of a direct introduction of partially methylated ethanolamine in the phospholipide molecule is accepted, such atypical phospholipides may be partly adsorbed on MgO and thus give higher values in the cephalin fraction. It is obvious that the speculations outlined above are merely working hypotheses and that a number of alternative explanations may be suggested.

SUMMARY

Rats maintained on a low casein, low fat diet were given by stomach tube a single dose of ethanolamine, methylethanolamine, dimethylethanolamine, or choline. The controls received water. The animals were then injected with isotopic phosphate and the radioactivity and the phosphorus content determined in the lipides of the liver and small intestine.

All the substances tested stimulated the formation of total phospholipides in both tissues. The stimulation by ethanolamine and by the products of its partial methylation was of the same order of magnitude or greater than that observed after choline was given.

In a number of experiments the liver phospholipides have been separated into choline-containing and non-choline-containing by adsorption on magnesium oxide. Under the conditions of the present experiments, the adequacy of the separation and the significance of the results obtained on the separated fractions are somewhat doubtful. To the extent of their reliability, these results showed that after ethanolamine, methyl-, and dimethylethanolamine were given, the radioactivity was increased in both phospholipide fractions, but the extent of the relative increase in each fraction varied with the substance administered. After choline was given, the increase was confined to the choline-containing phospholipides.

BIBLIOGRAPHY

1. Artom, C., and Cornatzer, W. E., *J. Elisha Mitchell Sc. Soc.*, **63**, 93 (1947); *Abstr. Communicat. XVII Internat., Physiol. Cong.*, Oxford, 56 (1947); *Federation Proc.*, **7**, 143 (1948).
2. Perlman, I., and Chaikoff, I. L., *J. Biol. Chem.*, **127**, 211 (1939).
3. Artom, C., and Cornatzer, W. E., *J. Biol. Chem.*, **171**, 779 (1947).
4. Artom, C., and Cornatzer, W. E., *J. Biol. Chem.*, **165**, 393 (1946).
5. Stetten, D., Jr., *J. Biol. Chem.*, **140**, 143 (1941).
6. Badger, E., *J. Biol. Chem.*, **153**, 183 (1944).
7. du Vigneaud, V., Chandler, J. P., Simmonds, S., Moyer, A. W., and Cohn, M., *J. Biol. Chem.*, **164**, 603 (1946).
8. Jukes, T. H., Oleson, J. J., and Dornbush, A. C., *J. Nutr.*, **30**, 219 (1945).
9. Jukes, T. H., Dornbush, A. C., and Oleson, J. J., *Federation Proc.*, **4**, 157 (1945).
10. Horowitz, N. H., Bonner, D., and Houlahan, M. B., *J. Biol. Chem.*, **159**, 145 (1945).
11. Jukes, T. H., and Dornbush, A. C., *Proc. Soc. Exp. Biol. and Med.*, **58**, 142 (1945).
12. Jukes, T. H., *J. Nutr.*, **22**, 315 (1941).
13. Horowitz, N. H., and Beadle, G. W., *J. Biol. Chem.*, **150**, 325 (1943).
14. Platt, A. P., and Porter, R. R., *Nature*, **160**, 905 (1947).
15. Fishman, W. H., and Artom, C., *J. Biol. Chem.*, **154**, 117 (1944).
16. Taurog, A., Entenman, C., Fries, B. A., and Chaikoff, I. L., *J. Biol. Chem.*, **155**, 19 (1944).
17. Artom, C., and Fishman, W. H., *J. Biol. Chem.*, **148**, 405 (1943).
18. Fisher, R. A., Statistical methods for research workers, London, 6th edition (1936).
19. Perlman, I., and Chaikoff, I. L., *J. Biol. Chem.*, **130**, 593 (1939).
20. Perlman, I., Stillman, N., and Chaikoff, I. L., *J. Biol. Chem.*, **133**, 651 (1940).
21. Best, C. H., Hershey, J. M., and Huntsman, M. E., *Am. J. Physiol.*, **101**, 7 (1932).
22. Platt, A. P., *Biochem. J.*, **33**, 505 (1939).
23. Fishman, W. H., and Artom, C., *J. Biol. Chem.*, **154**, 109 (1944).

24. Abdon, N. O., and Borglin, N. E., *Acta pharmacol. et toxicol.*, **3**, 73 (1947). Borglin, N. E., *Acta pharmacol. et toxicol.*, **3**, suppl. 1 (1947).
25. Entenman, C., Chaikoff, I. L., and Friedlander, H. D., *J. Biol. Chem.*, **162**, 111 (1946).
26. Bernheim, F., and Bernheim, M. L. C., *Am. J. Physiol.*, **104**, 438 (1933); **121**, 55 (1938).
27. Mann, P. J. G., and Quastel, J. H., *Biochem. J.*, **31**, 869 (1937).
28. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **169**, 247 (1947).

THE EFFECT OF CYSTEINE, HISTIDINE, AND METHIONINE ON THE PRODUCTION OF POLYCYTHEMIA BY COBALT*

By JAMES M. ORTEN and MARY C. BUCCIERO

(*From the Department of Physiological Chemistry, Wayne University College of Medicine, Detroit*)

(Received for publication, July 23, 1948)

The fact that cobalt administered daily in small amounts either orally or parenterally will produce a polycythemia is now well established. The polycythemia has been produced in a number of species of animals, including the rat, mouse, rabbit, dog, duck, and frog, and is characterized by an increase in the erythrocyte count and hemoglobin and hematocrit values without any significant alteration, either quantitatively or qualitatively, in the leucocytes (1). There is a distinct increase in the total blood volume due to an increase in the number of circulating erythrocytes, the plasma volume remaining essentially unaltered (2, 3).

The mechanism involved in the production of polycythemia by cobalt has received some attention. Previous work in this laboratory has indicated that there is some active stimulus to erythropoiesis, since a distinct reticulocytosis precedes the rise in the erythrocyte count (4). Barron and Barron (5) have suggested that cobalt may inhibit cellular respiration and thus produce a compensatory polycythemia for the purpose of increasing oxygen transport to the cells. In support of this hypothesis they have reported that the administration of ascorbic acid, allegedly involved in cell respiration, inhibits the production of polycythemia by cobalt in the rabbit. Other studies in this laboratory (6) add some indirect support to such a hypothesis by demonstrating that cobalt does not alter the oxygen-carrying capacity of hemoglobin nor does it form a "methemoglobin" in the rat. Thus if cobalt produces a compensatory polycythemia by interfering with the respiratory process, it must be the internal or cellular respiration which is affected rather than the external respiratory process.

The effect of several nitrogenous compounds on the action of administered cobalt in the animal organism has been investigated. Davis (7) reported that choline administered orally to dogs will completely inhibit the production of polycythemia by cobalt. This observation was not confirmed in this laboratory with the rat as the experimental animal (8). Griffith and his coworkers (9) have observed that cysteine, and to a lesser extent cystine, but not methionine, will greatly decrease the toxicity of orally administered cobalt in the rat as evidenced by growth response. However, they did not study the possible effects on hematopoiesis. These investigators attributed the toxic effect of cobalt to a "fixation of sulfhydryl compounds in tissues with resulting interference with oxidative mechanisms."

---

* A portion of the data in this paper was taken from a dissertation presented by Mary C. Bucciero in partial fulfilment of the requirements for the degree of Master of Science, Wayne University, 1948.

Preliminary reports were given before the American Society of Biological Chemists at Chicago, May, 1947, and at Atlantic City, March, 1948.

Another amino acid, histidine, has been investigated in connection with the toxicity of cobalt. Burk *et al.* (10) have observed that histidine decreases the toxicity of cobalt in certain bacteria and increases the growth and respiration of cobalt-treated microorganisms and cultures of various animal tissues. Burk *et al.* (11), as well as Michaelis (12), have found that cobalt forms a complex salt with histidine which combines irreversibly with oxygen. Thus, he also attributes the toxic effect of cobalt to an inhibition of cellular oxidation, perhaps by the formation of an oxygen-binding cobalt-histidine complex in the cell.

The purpose of the present investigation was to determine the possible effects of three of the previously mentioned substances, cysteine, methionine, and histidine, on the production of polycythemia by cobalt.

EXPERIMENTAL

Weanling, male, albino rats of the Connecticut Agricultural Experimental Station strain, weighing 40 to 50 gm., were used. They were housed in individual cages and were fed a synthetic basal ration having the following percentage composition: casein 20.0, sucrose 10.0, white corn dextrin 40.0, Crisco 25.7, Wesson's (13) salt mixture 4.0. Synthetic vitamin supplements were incorporated in the foregoing basal diet in the following amounts (in mg. per 100 gm. of diet): thiamine 1, riboflavin 2, pyridoxine 1, niacinamide 2, calcium pantothenate 4, inositol 200, *p*-aminobenzoic acid 60, folic acid 2, biotin 0.001, and 2-methyl-1,4-naphthoquinone 0.4. In addition, vitamins A, D, and E were supplied as haliver oil with viosterol fortified with α-tocopherol (100 mg. per 50 cc. of oil). 3 drops were administered individually to each rat twice weekly.

The rats were divided into five groups of ten animals each and were given supplements to the basal diet (per kilo of diet) as follows: (1) control, unsupplemented basal diet; (2) cobalt only (0.477 gm. of recrystallized $CoSO_4 \cdot 7H_2O$); (3) cobalt plus cysteine (1.56 gm. or 4.68 gm. of L-cysteine hydrochloride); (4) cobalt plus histidine (6.25 gm. of L-histidine monohydrochloride); and (5) cobalt plus methionine 4.44 gm. of DL-methionine). The lower level of cysteine, 1.56 gm. per kilo of diet, is that found by Griffith and coworkers (9) to neutralize the toxic effect of cobalt (equivalent to 0.477 gm. of $CoSO_4 \cdot 7H_2O$ per kilo of diet) in so far as response in body weight was concerned. The higher level of cysteine, 4.68 gm. per kilo of diet or 3 times the lower level, was used to determine whether a further effect might be obtained from the increased amount. The levels of methionine and of histidine are isomolar with the higher level of cysteine. All animals, with the exception of the controls, received cobalt as cobalt sulfate in an amount (0.477 gm. per kilo of diet) providing approximately 1.0 mg. of cobalt per rat per day, an amount found in earlier studies capable of producing a definite polycythemia.

In order to evaluate the possible effect of cysteine and histidine on the absorption of cobalt from the gastrointestinal tract, as will be discussed later, three additional groups of rats, of ten to fifteen animals each, in which cobalt with cysteine or histidine was given parenterally, were studied. One group (control) was injected with approximately 0.5 mg. of cobalt daily (1 cc. of an aqueous solution containing 250 mg. of recrystallized $CoSO_4 \cdot 7H_2O$ per 100 cc.). The second group was given an equivalent amount of cobalt as the cobalt-cysteine complex (1 cc. of a solution containing 250 mg. of $CoSO_4 \cdot 7H_2O$ + 500 mg. of L-cysteine hydrochloride + 240 mg. of $NaHCO_3$ per 100 cc.). The third group was given the same amount of cobalt as the cobalt-histidine complex (1 cc. of a solution containing 250 mg. of $CoSO_4 \cdot 7H_2O$ + 400 mg. of L-histidine monohydrochloride + 175 mg. of $NaHCO_3$ per 100 cc.). The solutions were injected subcutaneously in each case. The dosage of cobalt employed in these groups, 0.5 mg. of Co per day, was purposely reduced to approximately half that given orally to the preceding groups in order to compensate, partially at least, for the poor absorption of cobalt from the gastrointestinal tract (14). The amount of cysteine hydrochloride used in the preparation of the cobalt-cysteine complex is slightly in excess of the ratio 1:3, which Michaelis and Barron (15) have shown to be the combining ratio in the complex. Similarly, the amount of histidine monohydrochloride used in preparing the cobalt-histidine complex was slightly in excess of that required for a ratio of 1 part of cobalt to 2 of histidine (Michaelis (12)). In each case, the amount of $NaHCO_3$ added was slightly in excess of that needed to neutralize the hydrochloride of the amino acid preparation used. This also served to adjust the pH of the solutions to values at which the desired cobalt complexes form and at which irritation of the tissues by the injected solution was minimized.

Body weights and food consumption were determined weekly on the various groups of animals, and hemoglobin levels were determined biweekly. Hemoglobin was determined on blood obtained from a tail vein by an acid-hematin method with the Coleman spectrophotometer, previously calibrated by the oxygen capacity method. The animals were followed for periods varying from 12 to 20 weeks, as stated in Tables I and II.

RESULTS AND DISCUSSION

The results obtained are summarized in Tables I to IV. Table I gives the average body weight for the five groups of rats given oral supplementation. It is evident that the control rats fed the synthetic basal ration grew at a satisfactory rate. The animals given cobalt, on the other hand, showed an inhibition of growth, reaching an average weight of only 347 gm. in 20 weeks as compared to 542 gm. for the controls. The administration of either cysteine, histidine, or methionine with cobalt improved the growth rate considerably, although it was still less than that of the

Table I

Average Body Weights in Gm. of Control Rats and of Rats Fed Cobalt Alone or Supplemented with Cysteine, Histidine, or Methionine

Group (10 rats each)	Initial weight	Wks. on experiment										
		1	2	4	6	8	10	12	14	16	18	20
Control	44	110	162	258	325	376	420	463	493	517	526	542
Cobalt	45	77	98	135	176	205	237	269	289	309	326	347
" + cysteine												
Low level	42	70	110	166	218	256	298	333	364	380	374	395
High level	53	67	91	163	237	289	334	370*				
Cobalt + histidine	43	66	98	155	199	235	280	306	326	347	366	382
" + methionine	42	92	134	218	261	293	333	359	375	394	410	425

* Group discontinued.

Table II

Average Hemoglobin (Gm. Per Cent) Values for Control Rats and for Rats Fed Cobalt Alone or Supplemented with Cysteine, Histidine, or Methionine

Group	Initial	Wks. on experiment									
		2	4	6	8	10	12	14	16	18	20
Control	10.5	12.8	13.3	14.7	14.8	14.8	15.2	15.7	15.6	15.6	15.5
Cobalt	11.5	12.5	15.5	16.0	17.3	18.6	19.0	19.6	19.6	19.7	20.4
" + cysteine											
Low level	10.6	14.4	15.7	15.7	16.5	17.3	17.6	17.0	17.7	17.6	17.3
High level	10.9	13.5	14.3	15.0	16.3	16.7	17.1*				
Cobalt + histidine	11.3	14.0	16.0	16.5	16.8	17.9	18.2	17.9	18.2	18.2	18.2
" + methionine	10.5	12.9	16.6	17.2	17.5	19.2	19.8	19.5	19.9	19.9	19.9

* Group discontinued.

control animals. The rats given the higher level of cysteine showed somewhat higher average body weight during the 12 weeks they were observed than did the animals receiving the lower dosage. Such a result was not unexpected.

The average data for the daily food consumption (not given in the tables) for the various groups, indicated that the cobalt-treated rats ate more food per 100 gm. of body weight than did the control animals. The average group values for the 20th week of the study were as follows (gm. of food intake per day per 100 gm. of body weight): controls 2.5, cobalt only 3.4, cobalt + cysteine 3.2, cobalt + histidine 3.6, cobalt + methionine 3.2. These data suggest that cobalt decreases the retention or utilization of some dietary constituent or constituents, since the animals ingested more food per unit (100 gm.) of body weight. An improvement in "food utilization" evidently occurred in the rats given either cysteine, methionine, or histidine as a supplement to cobalt. It is interesting that the above values for food intake correspond, inversely, with the terminal average body weights shown in Table I.

TABLE III

*Statistical Analysis of Hemoglobin Data**

Group	Average hemoglobin	Standard deviation	Probable error of mean	Probable error† of difference beween means
Control	15.6	±0.17	±0.08	
Cobalt	19.7	±1.23	±0.24	
" + cysteine, low level	17.6	±1.60	±0.32	±0.40
" + histidine	18.2	±1.11	±0.23	±0.33
" + methionine	19.9	±1.23	±0.25	±0.35

* The values are those for the 18th week of the experiment.

† Comparison made with group given cobalt alone as the supplement.

Table II gives the average biweekly hemoglobin values. The controls show the normal progressive increase with age, reaching a constant adult level of about 15.6 gm. per cent. The cobalt-fed rats, on the other hand, developed a typical polycythemia as evidenced by the final hemoglobin value of 20.4 gm. per cent. The addition of cysteine, particularly at the higher level, lessened the increase of the hemoglobin values above the controls, as did histidine to a lesser extent. Methionine, on the other hand, had no noticeable effect. This was rather surprising in view of the favorable effect of methionine on the growth and food utilization of the cobalt-fed animals. A statistical analysis of the data, Table III, shows that the effects of cysteine and, to a lesser extent, of histidine, are highly significant, whereas that of methionine is not, as is also evident from a gross inspection of the data.

Since cobalt forms insoluble complexes with cysteine and with histidine, it appeared possible that these two substances might prevent the production of polycythemia by cobalt by merely decreasing the absorption of cobalt from the gastrointestinal tract. Therefore, three additional groups of rats were studied. They received subcutaneously 0.5 mg. of cobalt, either as cobalt sulfate or as the cobalt-cysteine or cobalt-histidine complex each day for a period of 12 weeks. As was found in the group of rats given oral supplementation, the injection of cobalt sulfate decreased the rate of growth (data not included) as compared with that of the controls. Much less inhibition of growth was observed in the groups given the cobalt-cysteine or cobalt-histidine complex, particularly the former. The data on hemoglobin are recorded in Table IV. It is evident that injected cobalt sulfate produced a polycythemia, whereas the cobalt-cysteine complex in an equivalent amount of cobalt did not increase the hemoglobin level significantly above that of the controls. The average hemoglobin values of the group receiving the cobalt-histidine complex, on the other hand, differed little from that to which cobalt sulfate was given and a typical polycythemia resulted. This observation is in general agreement with that in the group given cobalt with histidine orally, although some inhibition of the production of polycythemia by cobalt was observed in the latter case. This may be due to the fact that the ratio of histidine to cobalt was much greater in the orally supplemented group than was possible in the injected group. These data therefore indicate that the inhibition of the polycythemia by cysteine because of a possible impairment of cobalt absorption from the gastrointestinal tract cannot be a determining factor, since cobalt bound as a cysteine complex and administered parenterally in an amount comparable to that given orally likewise does *not* produce a polycythemia.

TABLE IV

Average Hemoglobin Values for Controls and Cobalt-Injected Rats

Group	No. of rats	Wks. on experiment						
		0	2	4	6	8*	10	12 (standard deviation)
Control	10	13.3	14.7	14.8	15.2	15.7	15.6	15.6 ± 0.17
$CoSO_4$	10	13.5	16.5	18.9	20.1	18.6	18.7	19.5 ± 0.97
Cobalt-cysteine complex	10	14.3	14.8	15.3	15.3	15.6	16.0	16.1 ± 1.11
Cobalt-histidine complex	15	13.2	16.5†	18.7†	20.0†	18.2	18.5	19.4 ± 0.82

* Daily injections omitted for 5 days during the 7th week.

† The cobalt-histidine complex solution injected during this interval did not contain added $NaHCO_3$, as described for the preparation of this solution in the text.

At least two explanations of the effect of cysteine and, to a lesser extent, histidine in preventing the production of polycythemia by cobalt appear possible. One is that these two substances, by forming complex compounds, may increase the excretion of cobalt and thus lessen its ability to produce polycythemia. However, such an explanation seems rather unlikely, because other substances such as choline and, under certain conditions, methionine also may form complexes with cobalt analogous to those of cysteine and histidine and thus presumably likewise increase cobalt excretion in the urine. Furthermore, under ordinary circumstances the excretion of parenterally administered cobalt is rapid and almost complete within 36 hours (14). Moreover, Stare and Elvehjem (16) have shown by analysis that at the height of the polycythemia there are present only 40 to 50 γ of cobalt in the entire body of the rat.

Another explanation, the more likely in our opinion, is that the administered cysteine, and possibly also histidine, combines with cobalt to form insoluble or inert complexes in the organism, thus preventing its subsequent "blocking" of sulfhydryl and perhaps other groups active in cellular respiration, which, in turn, would prevent the development of a compensatory polycythemia. Such an interpretation is in accord with the observation (Burk, personal communication) that "the relative affinities of cobalt for the naturally occurring amino acids are, in decreasing order, cysteine, histidine, and then the others." Cobalt also has a relatively low affinity for choline. This would thus satisfactorily explain the failure of methionine and choline to prevent the production of polycythemia by cobalt. However, further work will be required to answer these questions in a positive manner.

SUMMARY

The oral administration of 1.0 mg. of cobalt as cobalt sulfate to rats fed a synthetic basal diet produces a sustained polycythemia, an inhibition of growth, and impairment in food utilization.

Supplementation of the cobalt-containing diet with cysteine inhibits the production of the polycythemia. Histidine has a similar effect but to a lesser extent. Methionine has no detectable effect when fed in equivalent amounts.

Parenterally administered cobalt sulfate (0.5 mg. of cobalt per day) likewise produces a marked polycythemia, whereas an equivalent amount of cobalt as the cobalt-cysteine complex does not. Histidine injected with cobalt as the cobalt-histidine complex has less effect in preventing the development of polycythemia.

It is proposed that cysteine inhibits the production of polycythemia by cobalt by the formation *in vivo* of an inert cobalt-cysteine complex. Histi-

dine may act in a similar manner but the cobalt-histidine complex is more active (less completely formed) than the cobalt-cysteine complex at the pH values existing in the animal organism.

The suggestion is made that cobalt may produce polycythemia by binding sulfhydryl or perhaps other groups active in cellular respiration, thus leading to a simulated cellular anoxia and, in turn, to a compensatory polycythemia.

BIBLIOGRAPHY

1. Orten, J. M., Underhill, F. A., Mugrage, E. R., and Lewis, R. C., *J. Biol. Chem.*' **96**, 11 (1932).
2. Orten, J. M., Underhill, F. A., Mugrage, E. R., and Lewis, R. C., *J. Biol. Chem.*, **99**, 457 (1932–33).
3. Stanley, A. J., Hopps, H. C., and Hellbaum, A. A., *Proc. Soc. Exp. Biol. and Med.*, **61**, 130 (1946).
4. Orten, J. M., *Am. J. Physiol.*, **114**, 414 (1936).
5. Barron, A. G., and Barron, E. S. G., *Proc. Soc. Exp. Biol. and Med.*, **35**, 407 (1936).
6. Bucciero, M. C., and Orten, J. M., *Blood*, in press.
7. Davis, J. E., *Proc. Soc. Exp. Biol. and Med.*, **40**, 445 (1939).
8. Bucciero, M. C., and Orten, J. M., *Am. J. Physiol.*, in press.
9. Griffith, W. H., Pavcek, P. L., and Mulford, D. J., *J. Nutr.*, **23**, 603 (1942).
10. Burk, D., Schade, A. L., Hesselbach, M. L., and Fischer, C. E., *Federation Proc.*, **5**, 126 (1946).
11. Burk, D., Hearon, J., Caroline, L., and Schade, A. L., *J. Biol. Chem.*, **165**, 723 (1946).
12. Michaelis, L., *Arch. Biochem.*, **14**, 17 (1947).
13. Wesson, L. G., *Science*, **75**, 339 (1932).
14. Comar, C. L., and Davis, G. K., *Arch. Biochem.*, **12**, 257 (1947).
15. Michaelis, L., and Barron, E. S. G., *J. Biol. Chem.*, **83**, 191 (1929).
16. Stare, F. J., and Elvehjem, C. A., *J. Biol. Chem.*, **99**, 473 (1932–33).

THE USE OF ION EXCHANGE RESINS IN THE ISOLATION OF BLOOD GROUP A-SPECIFIC SUBSTANCE FROM HOG GASTRIC MUCIN*

By EDWARD L. BENNETT and CARL NIEMANN

(*From the Gates and Crellin Laboratories of Chemistry,† California Institute of Technology, Pasadena*)

(Received for publication, July 16, 1948)

Ion exchange resins (1) have been used in the investigation of A substance hydrolysates (2), but apparently no attempt has been made to determine whether they can be profitably used in the isolation or purification of undegraded A substance[1] from sources such as hog gastric mucin. Studies along these lines are reported in this communication.

EXPERIMENTAL

Ion Exchange Resins—De-Acidite (The Permutit Company, New York), washed on a 40 mesh screen with distilled water, was treated several times with aqueous 4 per cent sodium carbonate and repeatedly washed with distilled water, by suspension and decantation, until the supernatant liquid was colorless and had a pH of 8.0 or less. The resin collected on a suction filter contained 70 to 80 per cent water. 1 gm. (dry weight) of the resin contributed less than 0.05 milliequivalent of base to 75 ml. of distilled water when the suspension was allowed to stand, with frequent shaking, for 2 days at 25°. Under the same conditions 1 gm. (dry weight) of the resin removed 95 per cent of the hydrochloric acid from 75 ml. of a 0.066 M solution and 85 per cent from 75 ml. of a 0.093 M solution.

Amberlite IR-4 (The Resinous Products and Chemical Company, Philadelphia) prepared as described above, contained approximately 35 per cent water, and 1 gm. (dry weight) of the resin removed more than 95 per cent of the hydrochloric acid present in 25 ml. of a 0.166 M solution.

Amberlite IR-100 (The Resinous Products and Chemical Company), treated with 1 per cent hydrochloric acid and washed free of chloride, gave a product containing approximately 35 per cent water.

A Substance Preparations—A 2 per cent suspension of hog gastric mucin granules (Wilson), adjusted to pH 4.4 to 4.5 with glacial acetic acid, was

---

* This work was supported in part by a grant from the United States Public Health Service.

† Contribution No. 1225.

[1] Defined as a substance effective in inhibiting isoagglutination of human blood group A cells by group B serum and also effective in inhibiting lysis of sheep erythrocytes by human blood group A cell immune rabbit sera.

centrifuged twice in the open bowl of a Sharples centrifuge at 20,000 R.P.M. The centrifugate was used either directly or after ethanol fractionation (3, 4).

Procedure

Sufficient exchange resin was added, unless otherwise indicated, to a 1 to 2 per cent aqueous solution of the A substance preparation to provide 4 to 5 gm. (wet weight) of freshly washed resin for each gm. of dissolved solid. The suspension was stirred for 2 to 3 hours at 5°, filtered, and the operation repeated. The resulting solution was either lyophilized or subjected to further treatment with a different exchange resin. Equivalent N-acetylglucosamine contents and inhibition of hemolysis titers were determined as described previously (4, 5). Titration curves were determined by adding sufficient hydrochloric acid to 50 mg. of solid in 5 ml. of water to decrease the pH to 3.0 or less and then titrating with 0.040 M sodium hydroxide. Since an inflection point was observed at pH 8, the data are plotted in Fig. 1 with reference to the number of millimoles of acid or base required to change the pH of the above solution from pH 8 to some other pH.

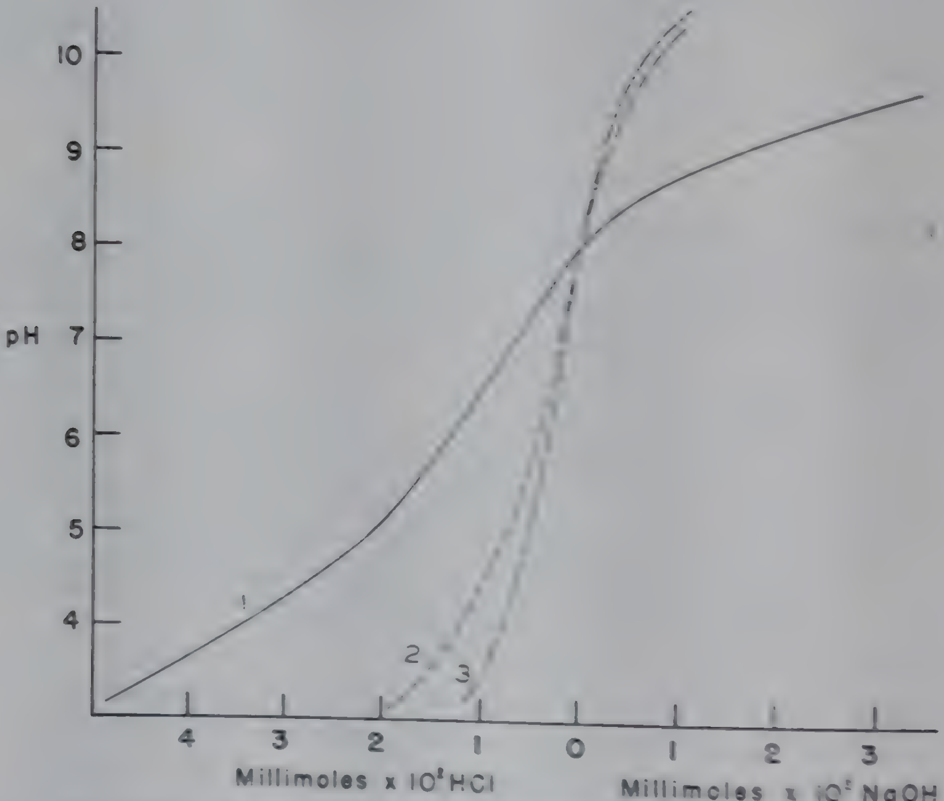


FIG. 1. Titration curves of A substance preparations. Curve 1, Fraction 136; Curve 2, Fraction 128; Curve 3, Fraction 141.

DISCUSSION

Within limits the equivalent N-acetylglucosamine content (5) of an A substance preparation can be taken as an index of the A substance activity of the preparation, evaluated in terms of inhibition of isoagglutination or inhibition of hemolysis (4, 5). Preliminary experiments based upon the determination of the equivalent N-acetylglucosamine content of an A substance solution before and after treatment with a solid adsorbent previously equilibrated with water indicated that of a number of adsorbents tested the anion exchange resins De-Acidite and IR-4 and the cation exchange resin IR-100 were sufficiently promising to warrant further investigation.[2]

The results obtained by treating an aqueous solution of hog gastric mucin with the above ion exchange resins, either singly or in combination, are given in Table I. It is noteworthy that successive application of De-Acidite and IR-100 gave a preparation which was as active in the hemolysis test[3] as those obtained from mucin by ethanol fractionation (3, 4). The separation of a resin-treated mucin solution into a clear supernatant and a less active viscous precipitate is reminiscent of the behavior observed when A substance preparations obtained from mucin by ethanol fractionation are electrodialyzed (4).[4] Although resin treatment will give products[3] as active as those obtained by ethanol fractionation, there is no evidence that further treatment of the product with any of the above resins will lead to more active preparations such as those obtained by a combination of ethanol fractionation and electrodialysis.[4]

A substance preparations of varying purity, obtained from hog gastric mucin by ethanol fractionation or electrodialysis, were subjected to resin treatment and, in contrast to the experience with mucin, only in one instance was an increase in activity observed (Table II). In the isolation of A substance from hog gastric mucin, it has been noted that an increase in A substance activity is usually accompanied by an increase in the equivalent N-acetylglucosamine content of the preparation (4).[4] However, with A substance preparations containing more than 10 per cent equivalent N-acetylglucosamine, the equivalent N-acetylglucosamine content may not necessarily increase with A substance activity upon further purification and in some instances may actually decrease.[4] This behavior is not unexpected, since it has been pointed out that blood group-specific substances other than the A substance contain alkali-labile bonds involving N-acetylglucosamine

---

[2] It is possible that with a different test or under different conditions other adsorbents may have been selected.

[3] Comparable titers were also obtained with the inhibition of the isoagglutination test which would indicate that resin treatment does not cause the degradation of A substance.

[4] Holzman, G., and Niemann, C., unpublished experiments.

TABLE I

Inhibition of Hemolysis Titer and Equivalent N-Acetylglucosamine Content of Hog Gastric Mucin Suspension after Successive Treatments with Several Exchange Resins

Description of fraction*	Fraction No. of product	Yield	Inhibition of hemolysis titer†	Equivalent N-acetylglucosamine content‡
Aqueous suspension of hog gastric mucin granules centrifuged twice at pH 4.4; centrifugate	135	72% from mucin	0.19 ± 0.02	7.6
Fraction 135 treated twice with De-Acidite	136	83% from No. 135	0.16 ± 0.01	8.9
Fraction 136 treated twice with IR-100; clear supernatant and viscous ppt. obtained upon standing 24 hrs.; separated	137 (ppt.)	50% from No. 136	0.11 ± 0.01	10.8
	138 (supernatant)	23% from No. 136	0.105 ± 0.005	11.6
Fraction 137 treated twice with IR-4	139	100% from No. 137		11.2
Fraction 138 treated twice with IR-4	140	100% from No. 138		11.7
Fraction 136 treated twice with IR-100 and twice with IR-4	141	67% from No. 136	0.12 ± 0.01	11.2
Fraction 135 treated alternately 4 times with De-Acidite and IR-100; allowed to stand 4 days; turbid supernatant and viscous ppt. obtained; separated	152 (supernatant)	32% from mucin	0.14 ± 0.02	10.5
	154 (ppt.)	20% from mucin	0.19 ± 0.02	9.2
Fraction 152 treated with De-Acidite and IR-100	153	88% from No. 152	0.12 ± 0.01	11.2

* 4 to 5 gm. (wet weight) of the exchange resin per gm. of material treated were used for each treatment, except in the preparation of Fractions 167 and 168, in which 8 gm. of De-Acidite per gm. were used for each treatment, and in the preparation of Fractions 152, 154, and 153, in which 0.5 gm. portions of De-Acidite and IR-100 per gm. were used for each treatment.

† Micrograms of test substance present in system in which sheep erythrocytes are 50 per cent hemolyzed by an anti-human A cell immune rabbit serum. The titers reported are the average of two or three determinations and are reported together with the average deviation of the several determinations.

‡ Expressed as equivalent per cent of N-acetylglucosamine in test substance (5). The analytical data reported are the mean results of duplicate or triplicate analyses. The absolute deviation was in no case larger than 0.3 per cent (equivalent N-acetylglucosamine).

(5, 6), and it is known that A- and O-specific substances can be separated, at least in part, by relatively simple fractionation procedures (7).[4]

TABLE II

Inhibition of Hemolysis Titer and Equivalent N-Acetylglucosamine Content of Some Blood Group A-Specific Substance Preparations after Treatment with Exchange Resins

Description of fraction*	Fraction No. of product	Yield	Inhibition of hemolysis titer†	Equivalent N-acetylglucosamine content‡
Aqueous suspension of hog gastric mucin granules centrifuged twice at pH 4.4; centrifugate (Fraction 135) fractionated with ethanol; material soluble in 40% (by volume) ethanol, insoluble in 65% (by volume) ethanol	143	22% from mucin	0.11 ± 0.01	10.6
Fraction 143 treated twice with De-Acidite	144	77% from No. 143	0.105 ± 0.015	12.0
Fraction 144 treated twice with IR-100	145	100% from No. 144	0.105 ± 0.010	12.3
Fraction 145 treated twice with IR-4	147	100% from No. 145	0.11 ± 0.01	12.1
Aqueous suspension of hog gastric mucin granules; centrifugate fractionated with ethanol; material soluble in 30% (by volume) ethanol, insoluble in 65% (by volume) ethanol; upon reprecipitation, insoluble in 45% (by volume) ethanol; dialyzed, then electrodialyzed	110		0.20 ± 0.02	11.8
Fraction 110 treated twice with De-Acidite	167	74% from No. 110	0.21 ± 0.02	13.4
Fraction 110 treated twice with IR-4	165	91% from No. 110	0.20 ± 0.02	11.8
Same as Fraction 110, except upon reprecipitation, material soluble in 45% (by volume) ethanol, insoluble in 65% (by volume) ethanol; dialyzed, then electrodialyzed	126		0.088 ± 0.005	10.4
Fraction 126 treated twice with De-Acidite	168	60% from No. 126	0.070 ± 0.005	11.9
Fraction 126 treated twice with IR-4	166	88% from No. 126	0.086 ± 0.005	10.5

See the corresponding foot-notes to Table I.

The effectiveness of De-Acidite and IR-100 in reducing the " buffering capacity" of mucin solutions and partially purified A substance preparations is clearly illustrated in Fig. 1. The anion exchange resin IR-4 was found to be markedly inferior to De-Acidite in this respect. Analysis of Fractions

135, 136, and 137 for total nitrogen, amino nitrogen, and amino acid nitrogen (Table III) revealed that De-Acidite was instrumental in removing acidic nitrogenous non-blood group-specific substances containing little or no amino nitrogen, whereas the substances removed by IR-100 contained substantial amounts of amino and amino acid nitrogen. At least part of the materials removed by De-Acidite are non-dialyzable.[5] It is interesting to note that in a centrifuged mucin solution approximately 20 per cent of the solids are not precipitated by 66 per cent ethanol, whereas approximately 30 per cent are removable by successive treatment with De-Acidite and IR-100.

It has been observed that many A substance preparations are contaminated by non-blood group-specific substances[6] exhibiting marked specific absorption in the 260 to 270 mμ region (8). An A substance preparation (Fraction 110) containing a substantial amount of the "260 mμ component" ($E_{1\text{ cm.}}^{1\%}$ at 260 mμ = 13.2) was treated with De-Acidite and the resulting preparation (Fraction 167) was found to have a value of $E_{1\text{ cm.}}^{1\%}$ at 260 mμ of 3.6. Treatment with IR-100 or IR-4 caused little or no decrease in extinction. The fact that De-Acidite was effective and IR-4 was relatively ineffective in removing the "260 mμ component" would indicate that the removal of this component by De-Acidite is not a simple anion exchange. An explanation of the mode of action of De-Acidite leading to the loss of the "260 mμ component," a gain in the equivalent N-acetylglucosamine content, and no significant change in A activity must await the accumulation of additional data.

Table III

Nitrogen Content of A Substance Preparations

Fraction No.	Total N	Amino N	Amino acid N
	per cent	*per cent*	*per cent*
135	8.4	1.9	1.0
136	7.3	2.0	1.2
137	6.4	0.16	0.09

SUMMARY

The treatment of hog gastric mucin with the two ion exchange resins De-Acidite and IR-100 has given apparently undegraded A substance preparations which are as active as those obtained from the same source by

---

[5] Bennett, E. L., unpublished data.

[6] While the "260 mμ component" has not been obtained free of A substance, all evidence (8) points to its non-blood group specific character.

ethanol fractionation. This enrichment in A substance is due to the partial removal by these two resins of some of the non-blood group-specific components normally present in hog gastric mucin.

The authors wish to express their indebtedness to Dr. D. H. Brown and Dr. G. Holzman for their assistance in this investigation.

BIBLIOGRAPHY

1. Kunin, R., *Ind. and Eng. Chem.*, **40**, 41 (1948).
2. Freudenberg, K., Walch, H., and Molter, H., *Naturwissenschaften*, **30**, 87 (1942).
3. Landsteiner, K., and Harte, R. A., *J. Exp. Med.*, **71**, 551 (1940).
4. Brown, D. H., Bennett, E. L., Holzman, G., and Niemann, C., *Arch. Biochem.*, **13**, 421 (1947).
5. Holzman, G., Bennett, E., Brown, D., and Niemann, C., *Arch. Biochem.*, **11**, 415 (1946).
6. Morgan, W. T. J., and Waddell, M. B. R., *Brit. J. Exp. Path.*, **26**, 387 (1945).
7. Aminoff, D., Morgan, W. T. J., and Watkins, W. M., *Nature*, **158**, 879 (1946).
8. Holzman, G., and Niemann, C., *J. Biol. Chem.*, **174**, 305 (1948).

STUDIES ON THE NATURALLY OCCURRING PENICILLINS

II. PRECIPITATION OF CRYSTALLINE AMMONIUM PENICILLINS

By CHARLES J. SALIVAR, THOMAS C. GRENFELL, and ELLIS V. BROWN*

(*From the Laboratories of Chas. Pfizer and Company, Inc., Brooklyn, New York*)

(Received for publication, December 5, 1946)

It has been disclosed (1) that there are several antibiotics of the penicillin class and that all are salts of acids having the empirical formula $C_9H_{11}O_4SN_2$—R. Among the naturally occurring penicillins are those in which R is respectively benzyl, *n*-heptyl, 2-pentenyl, and *p*-hydroxybenzyl. Commercial penicillin may be any one or a mixture of these penicillins, depending on the method of production and isolation. The alkali and alkaline earth salts of penicillin are extremely soluble in water. The only known methods of crystallizing penicillin salts such as sodium, potassium, and ammonium are by the use of organic solvents, usually in the presence of minute amounts of water. The penicillins, as usually isolated, are associated with colored and odoriferous materials which are sometimes very difficult to remove by simple methods.

We have found that by adding soluble ammonium salts to moderately concentrated solutions of various penicillin salts it is possible to crystallize a high proportion of the penicillin as ammonium penicillin. It is possible to prepare material with little color or odor by the procedure. The penicillin used should be in a concentration equivalent to at least 200,000 units per ml. as tested by the turbidimetric method with *Staphylococcus aureus* strain H (2). It should also have a quality of 1000 units per mg. or better when dried. With more crude or dilute starting material, the results are dependent on the nature and quality of the penicillin. In our hands, it has mattered little whether the original penicillin was of the benzyl, pentenyl, or heptyl species, as long as sufficient ammonium salt was added. With any given salt concentration, the solubilities of the various penicillin species differ considerably. This is illustrated in Table I, which gives the solubilities of ammonium benzyl- and heptylpenicillins in saturated ammonium chloride solution.

The ammonium salts which seem to be most useful are the sulfate, chloride, bromide, and acetate. However, a number of others have been used and a comparison of the yields of ammonium penicillin after addition of a solution of pure sodium benzylpenicillin having 300,000 units per ml. to various strong ammonium salt solutions is given in Table II.

---

* Present address, Fordham University, New York 58.

The ammonium penicillin precipitate is washed with fresh strong ammonium salt solution and is of course contaminated with this ammonium salt. When a pure ammonium penicillin is desired, we have found ammonium sulfate to be the salt of choice for the precipitation because of its easy removal. By treating the air-dried precipitate with the proper amount of dioxane containing 10 per cent water, the ammonium sulfate remains undissolved. After filtration, the solution is then treated with 4 volumes of dry dioxane to precipitate the pure ammonium penicillin. When ammonium acetate is used as the precipitant, the product may be dried under a high vacuum to remove the ammonium acetate and give a crystalline ammonium penicillin of high potency. As both ammonium chloride and bromide present some difficulty in their separation from ammonium penicillin, their use is limited to instances in which other salts are desired. In these cases, the crude ammonium salt is dissolved in water, acidified at 0–4°, and shaken with ether. The ether solution is then treated with an alkali or alkaline earth bicarbonate solution. On evaporating this aqueous solution in a high vacuum, a high quality penicillin salt is obtained which in many cases may be crystallized.

TABLE I

Solubilities of Ammonium Benzylpenicillin and Heptylpenicillin in Saturated Ammonium Chloride

Penicillin	Amount at start	In mother liquor	Insoluble	Heptyl-	Benzyl-	Solubility in saturated NH_4Cl
	mg.	*mg.*	*mg.*	*per cent*	*per cent*	*mg. per ml.*
Heptyl-..........	120	8	112	100	0	0.8
"	60	50	70	95	5	5
Benzyl-...........	60					
Heptyl-..........	60	80	40	Not determined		8
Benzyl-...........	120					
Heptyl-..........	120	50	110	85	15	5
Benzyl-...........	60					
"	140	118	23	0	100	11.8

The method is being extended to the use of salts other than ammonium and shows considerable promise.

EXPERIMENTAL

In determining the solubilities of ammonium benzyl- and heptylpenicillins in saturated NH_4Cl (Table I) the amount of penicillin used was warmed to 40–45° with 10 ml. of saturated ammonium chloride (pH 6.8) and then allowed to stand at room temperature for 1 hour. After filtration, the insoluble material was dissolved in water and the amount of penicillin was determined by optical rotation. The same method was used on the soluble portion. For this determination $[\alpha]_D^{25} = +300° \pm 5°(c = 1 \text{ in water})$ was used as the value for pure sodium benzylpenicillin and $[\alpha]_D^{25} = +285° \pm 5°$ for the pure sodium heptylpenicillin. The proportion of insoluble ammonium benzylpenicillin was determined by means of the Beckman spectrophotometer (3).

For the determination of the relative precipitating action of the various ammonium salts (Table II) 1 gm. of pure sodium benzylpenicillin crystals was dissolved in 1 ml. of water, treated with 5 ml. of the concentrated salt solution with stirring, and the precipitate washed with 5 ml. of the same concentrated solution. The concentration of penicillin in the precipitate and in the mother liquor was determined by rotation and bioassay as previously discussed.

TABLE II

Precipitation of Ammonium Benzylpenicillin by Salts

Ammonium salt	Amount in ppt.	In mother liquor	Concentration of ammonium salt per 100 ml. solution
	mg.	*mg.*	*gm.*
Acetate	965	35	60
Bromide	709	291	55
Chloride	887	113	30
Formate	980	20	70.4
Iodide	0	1000*	83.3
Nitrate	805	195	68.6
Phosphate	980	20	46
Sulfate	998	2	53

All solutions adjusted to pH 6.5 to 7.5 with NH_3 or the corresponding acid.

* Decomposition was very rapid and a slight precipitate dissolved in a short time.

When 262 ml. of saturated $(NH_4)_2SO_4$ solution, adjusted to pH 6.5 with NH_4OH, were treated, with stirring, with 60 gm. of commercial sodium penicillin assaying 1100 units per mg., the penicillin ammonium salts soon began to crystallize and within a few minutes crystallization was complete. The magma was filtered and washed with 50 per cent $(NH_4)_2SO_4$ solution until the wash liquor became colorless. After drying in a vacuum oven, 44.1 gm. of product containing 78 per cent ammonium penicillins were obtained. This is 86 per cent of the penicillins contained in the starting material.

In another experiment 400 ml. of crude sodium penicillin liquor assaying

300,000 units per ml. were treated with 300 gm. of powdered $(NH_4)_2SO_4$ with stirring. The precipitate was filtered and washed as indicated previously. The dry cake was stirred with 400 ml. of dioxane containing 10 per cent of water. After filtration, the solution was treated with 1200 ml. of dry dioxane. The ammonium penicillin was precipitated and after filtering, washing, and drying 48.7 gm. of mixed ammonium penicillin were obtained. This was 67 per cent of the penicillin in the starting material. The product was shown to be essentially all ammonium penicillin by its rotation ($[\alpha]_D^{25} = +294°$ (c = 1 per cent in water)) and its biological assay of 1650 units per mg.

When a crude sodium penicillin solution containing essentially all of the penicillin as the benzyl species was treated in the same manner, there were obtained 12 gm. of ammonium salt from 100 ml. of liquor assaying 300,000 units per ml. This product was pure ammonium benzylpenicillin as shown by analysis, rotation, and bioassay. The optical rotation was $[\alpha]_D^{25} = +298°$ (c = 1 per cent in water) and the bioassay 1650 units per mg.

$C_{16}H_{21}O_4SN_2$. Calculated. C 54.70, H 5.98, N 11.97, S 9.12
Found. " 54.62, " 6.15, " 11.90, " 9.31

Ammonium acetate (172.5 gm.) was adjusted to pH 6.5 with NH_4OH and made up to 330 ml. with water. This solution was treated with 100 gm. of dry commercial penicillin assaying 1000 units per mg. Stirring was continued for about 15 minutes to crystallize the penicillin. The ammonium penicillin was then filtered and washed with 50 per cent ammonium acetate solution. On drying in a high vacuum, the excess ammonium acetate volatilized and 30.8 gm. of ammonium penicillin, having $[\alpha]_D^{25} = +296°$ (c = 1 per cent in water) and a bioassay of 1620 units per mg., were obtained. This is 50 per cent of the penicillin in the starting material.

To 770 ml. of a crude sodium benzylpenicillin concentrate containing 300,000 units per ml. were added 230 gm. of NH_4Cl with stirring. The mixture was filtered and washed with saturated ammonium chloride. The vacuum-dried cake contained 75 per cent of the original penicillin as crystalline ammonium salt contaminated with ammonium chloride. The crude ammonium salt was dissolved in 2 liters of water, adjusted to pH 2 with H_3PO_4, and extracted three times with 2 liter portions of ether. The ether extract was then stirred with 2 per cent $NaHCO_3$ solution. Additions of fresh $NaHCO_3$ solution were made until pH 7 was reached. This solution, on evaporation in a high vacuum, yielded amorphous sodium benzylpenicillin, which was dissolved in 400 ml. of 10 per cent aqueous dioxane and precipitated by the addition of 1200 ml. of dry dioxane. The resulting crystalline salt had $[\alpha]_D^{25} = +299°$ and a bioassay of 1660 units per mg.

$C_{16}H_{17}O_4N_2SNa$. Calculated. C 53.93, H 4.81, N 7.86, S 9.00, Na 6.46
Found. " 53.97, " 5.01, " 7.86, " 9.03, " 6.43

We wish to thank Dr. J. A. Means for performing the microanalyses. We also wish to acknowledge the interest and encouragement of Dr. Richard Pasternack and Mr. Howard Hedger.

SUMMARY

A new method for the purification of penicillin, which gives an essentially colorless, odorless ammonium penicillin, has been devised. Other pure salts may be made from the ammonium salts by known methods. The method appears to be independent of the species of penicillin present.

BIBLIOGRAPHY

1. Committee on Medical Research, Office of Scientific Research and Development, Washington, and the Medical Research Council, London, *Science*, **102**, 627 (1945).
2. McMahan, J. R., *J. Biol. Chem.*, **153**, 249 (1944).
3. Grenfell, T. C., Means, J. A., and Brown, E. V., *J. Biol. Chem.*, **170**, 527 (1947).

FRACTIONATION OF SERUM INTO ALBUMIN AND α-, β-, AND γ-GLOBULIN BY SODIUM SULFATE

By ANDRE C. KIBRICK and MURIEL BLONSTEIN

(From the Department of Chemistry, The Bronx Hospital, New York)

(Received for publication, July 2, 1948)

The values of the albumin and total globulin of serum determined with 22.4 per cent sodium sulfate by the method of Howe (1) are better correlated with the values of one fraction containing the albumin and α-globulin and of another containing the β- and γ-globulins (2). However, 19 per cent sodium sulfate seems to separate these fractions more efficiently, since it yields results which agree very well with the corresponding fractions determined by electrophoresis (3). Apparently the precipitation of all of the globulin fractions of human serum requires 26 to 27 per cent sodium sulfate (3, 4). The data of Majoor (4) indicate that most of the γ-globulin is precipitated by 15 per cent of the salt. This is consistent with the findings of Gutman *et al.* (5) that the γ-globulin fraction is only partially precipitated at a concentration of 13.5 per cent of the salt, while 17.4 per cent removes a significant amount of β-globulin in addition to the γ fraction. Moreover, 15 per cent sodium sulfate is approximately equivalent to 0.33 saturated ammonium sulfate in respect to salting out. Jager and Nickerson found a good correlation between the amounts of protein precipitated by the latter and the values of γ-globulin estimated by electrophoresis (6). Hence, it occurred to us that human serum can be analyzed for albumin and for all of the globulin fractions by determining the protein precipitated with 15, 19, and 26 per cent sodium sulfate. The present study was undertaken to determine whether the values estimated by this simple method are consistent with the results of other methods.

EXPERIMENTAL

A series of thirteen sera, obtained from patients with miscellaneous clinical conditions in this hospital, was analyzed by fractionation with sodium sulfate. In addition, the albumin and total globulin were determined with methyl alcohol by the method of Pillemer and Hutchinson (7), and the γ-globulin fraction was determined with serum of rabbits immunized to this protein. Another series of sera and plasmas of known protein composition determined by electrophoretic analysis was obtained from other laboratories.[1] These were analyzed by fractionation with salt.

---

[1] We wish to thank the following for samples of blood and for the results of the electrophoretic analyses: Dr. Mary L. Petermann and Dr. Nelson F. Young, Sloan-

The fractionation was made by adding 0.5 ml. of serum or plasma to 10 ml. of 15.75, 19.90, and 27.20 per cent sodium sulfate at 37°. About 10 mg. of Hyflo Super-Cel were added from a scoop made from glass tubing, and the mixtures were allowed to stand in the incubator at 37° for 1 hour.[2] The precipitates were then filtered in covered funnels in the incubator with Whatman No. 50 filter paper, 9 cm. in diameter. Portions of the clear filtrates were added to the biuret reagent, and their protein content was determined by the method of Kingsley (8) as modified by Kibrick and Clements (9).

The biuret reaction was also utilized to determine the total protein of the sera and the albumin in the methyl alcohol filtrates from the method of Pillemer and Hutchinson, as described previously (9).

Serum immune to human γ-globulin was obtained by injecting rabbits twice weekly by ear vein with 1 to 2 ml. of 1 per cent of the protein in glycine solution[3] containing 0.05 per cent aluminum ammonium sulfate. After about 6 weeks and a period of 6 days without injections, the animals were bled from the heart and the serum was prepared with 0.01 per cent sodium merthiolate. Traces of antibodies to the other serum proteins were removed by absorption with a solution of human albumin[4] and with a mixture of the α- and β-globulins.[5] The determinations were made by adding 2 ml. of the immune serum in centrifuge tubes to 1 ml. of human serum which had been diluted 50 to 150 times with 0.9 per cent sodium chloride. The tubes were allowed to stand in a water bath at 40° for 2 hours and then placed in the refrigerator until the next day. The immune precipitates were centrifuged and washed twice with 3 ml. of cold 0.9 per cent sodium chloride. They were then dissolved in dilute alkali and their content of

---

Kettering Institute, New York; Miss Miriam Reiner, Mount Sinai Hospital, New York; Dr. Dan H. Moore, Electrophoresis Laboratory, Columbia University. Some of the samples were citrated plasmas which had been frozen for storage and thawed out just before analysis. Others were plasmas prepared with a minimum of heparin. Although some of the fibrinogen had become insoluble and was removed, a significant amount remained in solution. This was of no consequence, however, since the γ-globulin precipitated with 15 per cent sodium sulfate was corrected for the residual fibrinogen indicated by the electrophoretic patterns.

[2] We have found that the amount of soluble protein was essentially the same when the precipitates were filtered from 30 minutes to as long as 24 hours after mixing. The filtrates were frequently turbid, however. The use of Super-Cel produces clear filtrates in almost every case and does not affect the results.

[3] Prepared from immune serum globulin, Cutter Laboratories, containing 17 per cent of the protein in 0.3 M glycine.

[4] We wish to thank Dr. F. F. Johnson of the Cutter Laboratories, Berkeley, for samples of human albumin and γ-globulin which do not contain other electrophoretic components.

[5] We are indebted to Professor E. J. Cohn for a concentrated fraction of the α- and β-globulins from human serum.

nitrogen was determined by the micro-Kjeldahl method. Several standard tubes containing a dilute solution of γ-globulin[4] were run with each series of determinations. The concentration of γ-globulin was calculated from a standard curve prepared from the results of serial dilutions of a weighed amount of the protein.

RESULTS AND DISCUSSION

Table I shows that the values of albumin and total globulin determined with sodium sulfate agree quite well with those determined with methyl alcohol. This is further confirmation that 26 per cent sodium sulfate effects a reliable separation in the serum of subjects with a variety of clinical conditions. The values of the γ-globulin fraction also agree quite well with the results obtained by precipitation with immune serum. Kabat *et al.* have determined the γ-globulin in cerebrospinal fluid by the latter method (10), but it does not seem so convenient for routine use. Table II shows that the protein precipitated with 15 per cent sodium sulfate is in satisfactory agreement with the γ-globulin fraction estimated by electrophoretic analysis.

TABLE I

Comparison between Values Derived from Fractionation with Sodium Sulfate and Those from Precipitation with Methyl Alcohol and with Immune Serum

The results are expressed in gm. per 100 ml.

Sample No.	Total protein	Albumin		Globulin fractions			
		Salt	Alcohol	With sodium sulfate			Precipitin
				α-	β-	γ-	γ-
1	7.16	3.74	3.57	1.04	0.61	1.77	1.6
2	6.98	3.87	4.02	0.75	0.82	1.54	1.7
3	6.98	3.23	3.42	1.30	0.66	1.79	1.8
4	6.70	3.71	3.63	0.70	1.05	1.24	1.2
5	4.78	1.79	1.21	0.93	0.56	1.5	1.2
6	8.00	3.99	4.12	0.86	0.68	2.47	2.5
7	7.49	3.93	4.25	0.99	0.61	1.96	2.2
8	8.14	3.07	2.95	1.05	0.76	3.26	3.0
9	7.04	3.44	3.71	1.18	0.57	1.85	1.5
10	7.04	3.24	3.48	1.51	0.77	1.52	1.3
11	7.15	3.43	3.21	0.83	0.47	2.42	2.3
12	7.95	3.70	3.86	1.25	0.70	2.30	2.2
13	4.02	1.02	1.38	1.35	0.57	1.08	1.3

We have found that the amount of protein precipitated with 19 per cent sodium sulfate in about 100 different sera is only slightly less than that precipitated with 22 per cent salt by the method of Howe. In many instances the results were almost identical, but there was a suggestion that better agreement is possible between duplicate determinations with the smaller concentration. There seems to be a definite break in the precipitation of protein at about 19 per cent, which the data of Milne (3) indicate is equal to the sum of the β- and γ-globulin fractions. If this is correct, the amounts of the α and β fractions, derived from this value and from the values of total globulin and of the γ fraction, are also correct. Table II shows that they are in agreement with those found by electrophoresis.

TABLE II

Comparison between Results of Fractionation with Sodium Sulfate and Those from Electrophoresis

The results are expressed in gm. per 100 ml.

Sample No.	Salt fractionation				Electrophoresis			
	Albumin	Globulin			Albumin	Globulin		
		α-	β-	γ-		α-	β-	γ-
1				0.8	3.2	0.8	1.1	0.9
2				5.3	1.7	0.8	0.5	4.5
3				0.6	3.8	1.0	0.8	0.7
4				1.0	3.4	1.4	1.2	1.2
5				1.2	4.5	1.2	1.0	1.2
6	2.8	1.0	1.1	1.6	3.0	0.9	1.0	1.5
7	2.8	1.0	1.2	1.2	3.0	0.8	1.0	1.3
8	2.8	1.0	0.9	1.5	2.8	0.8	1.0	1.6
9	2.8	0.8	1.2	1.6	2.7	0.7	1.2	1.7
10	2.3	1.3	0.7	1.1	2.5	1.1	0.7	0.9
11	3.0	1.3	0.7	0.8	3.3	1.0	0.7	0.8
12	2.4	1.3	1.0	0.9	2.4	1.5	1.0	0.7
13	2.3	1.1	1.0	1.0	2.2	1.2	0.9	0.7
14	2.1	0.9	0.7	0.7	2.2	0.8	0.6	0.7

We are grateful to Dr. Joseph Felsen, Director of Laboratories and Research, for advice and encouragement.

SUMMARY

1. A simple chemical method is proposed to estimate the fractions of protein in human serum by precipitation with 15, 19, and 26 per cent sodium sulfate. The results of a series of thirteen sera are compared with the values of albumin and total globulin determined with methyl alcohol and with the values of γ-globulin determined by precipitation with immune rabbit serum. The results in another series of fourteen samples are compared with values derived from electrophoretic analysis.

2. Most of the values of albumin, total globulin, and γ-globulin are within 0.2 gm. of those determined with methyl alcohol and with immune serum.

3. The values of albumin and of α-, β-, and γ-globulin also compare favorably with the results of electrophoresis.

BIBLIOGRAPHY

1. Howe, P. E., *J. Biol. Chem.*, **49**, 93 (1921).
2. Petermann, M. L., Young, N. F., and Hogness, K. R., *J. Biol. Chem.*, **169**, 379 (1947).
3. Milne, J., *J. Biol. Chem.*, **169**, 595 (1947).
4. Majoor, C. L. H., *J. Biol. Chem.*, **169**, 583 (1947).
5. Gutman, A. B., Moore, D. H., Gutman, E. B., McClellan, V., and Kabat, E. A., *J. Clin. Invest.*, **20**, 765 (1941).
6. Jager, B. V., and Nickerson, M., *J. Biol. Chem.*, **173**, 683 (1948).
7. Pillemer, L., and Hutchinson, M. C., *J. Biol. Chem.*, **158**, 299 (1945).
8. Kingsley, G. R., *J. Lab. and Clin. Med.*, **27**, 840 (1942).
9. Kibrick, A. C., and Clements, A. B., *J. Lab. and Clin. Med.*, **33**, 662 (1948).
10. Kabat, E. A., Glusman, M., and Knaub, V., *Federation Proc.*, **7**, 306 (1948).

LETTERS TO THE EDITORS

PUTRESCINE AS A GROWTH FACTOR FOR HEMOPHILUS PARAINFLUENZAE*

Sirs:

A requirement for unidentified growth factors by some members of the genus *Hemophilus* has been reported.[1] During a study of the nutritional requirements of one member of this genus, *Hemophilus parainfluenzae* 7901, an amino acid medium was developed which was satisfactory for the assay of an essential factor supplied by a variety of crude materials including yeast and orange juice. Orange juice was chosen for fractionation and a crystalline compound, identified as putrescine, was isolated which completely replaced orange juice when added to the amino acid medium. The isolation was accomplished by the following procedures: (1) adsorption from clarified canned orange juice on Amberlite IR-100-H; (2) elution from the cation exchange resin with 20 per cent HCl after removal of inactive solids with 4 per cent H_2SO_4; (3) concentration of the eluate to small volume, addition of 50 per cent NaOH, and steam distillation; (4) neutralization of steam distillate with 10 per cent H_3PO_4 and precipitation of the dipicrate by addition of sodium picrate solution to the concentrated steam distillate; and (5) repeated recrystallization from hot water. The dipicrate was converted to the dihydrochloride and the dibenzoate by standard procedures. The dibenzoate melted at 178–180°. A mixture with an authentic sample of putrescine dibenzoate (m.p. 178–180°) melted at 178–180°. The melting point of the dipicrate also checked that reported in the literature and gave no depression with an authentic sample of this compound.

Analysis of Dihydrochloride—$C_4H_{12}N_2 \cdot 2HCl$. Calculated, N .17.39; found, 17.43, 17.48.

The essential nature of putrescine for growth of *H. parainfluenzae* is shown in the table. Spermine and spermidine, whose molecules contain the 1,4-diaminobutane residue, are also active. However, a variety of compounds having a structural relationship to putrescine and including ornithine, cadaverine, 1,3-diaminopropane, 1,6-diaminohexane, *n*-butylamine, and pyrrolidine was found to be totally inactive, as were the polyamines, triethylenetetramine and tetraethylenepentamine. A high degree of specificity for the tetramethylenediamine structure is thus indicated.

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Williams-Waterman Fund of the Research Corporation.

[1] Bass, A., Berkman, S., Saunders, F., and Koser, S. A., *J. Infect. Dis.*, **68**, 175 (1941).

*Effect of Putrescine, Spermine, and Spermidine on Growth of Hemophilus parainfluenzae**

Orange juice solids		Putrescine		Spermine		Spermidine	
	Turbidity†		Turbidity†		Turbidity†		Turbidity†
mg. per 10 cc.		*γ per 10 cc.*		*γ per 10 cc.*		*γ per cc. 10*	
0	97	0	97	0	97	0	97
0.25	89	0.10	90	0.50	88	0.50	87
0.5	87	0.25	88	1.0	86	1.0	84
1.0	84	1.0	85	2.0	84	2.0	83
5.0	82	5.0	80	5.0	81	5.0	82
		10.0	80‡	10.0	81‡	10.0	82‡

* 10 cc. volumes incubated in Pyrex milk dilution bottles for 48 hours at 37°. The basal medium contains, per 10 cc., 2.5 cc. of amino acid solution,[2] 10 mg. of dextrose, 60 mg. of sodium acetate, 100 γ of guanine, adenine, and uracil, 1 γ of thiamine and riboflavin, 5 γ of nicotinic acid and nicotinamide, 20 γ of pyridoxine, 10 γ of calcium pantothenate, 0.01 γ of biotin and *p*-aminobenzoic acid, 0.1 γ of folic acid, 200 γ of inositol, 10 γ of Ca^{++} and Mg^{++}, 1 γ of Zn^{++}, Cu^{++}, Co^{++}, and Mn^{++}, 25 γ of Fe^{++}, 15.6 mg. of K_2HPO_4, and 1.4 mg. of KH_2PO_4. Initial pH, 7.8. Following sterilization of the medium, 1 γ of coenzyme I was added to each culture bottle. Putrescine was added as the dihydrochloride, spermidine and spermine as the phosphates. The additions are expressed in terms of the free bases.

† Per cent of incident light transmitted; uninoculated medium = 100.

‡ Growth in presence of 10 γ of the indicated compound plus 1 mg. of orange juice solids.

To our knowledge, this is the first demonstration of an essential nutritional function for one of the putrefactive amines, and indicates that putrescine and possibly additional compounds of this group play a much more important metabolic rôle than has been previously indicated.

Department of Biochemistry
College of Agriculture
University of Wisconsin
Madison

EDWARD J. HERBST
ESMOND E. SNELL

Received for publication, August 30, 1948

---

[2] Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172**, 15 (1948).

AN INTERRELATIONSHIP OF PURINES AND VITAMIN B_{12}

Sirs:

Thymidine has recently been reported to replace vitamin B_{12} in stimulating growth of *Lactobacillus lactis* Dorner.[1,2] We have found that purines (or their derivatives) and thymidine are essential for growth of *Lactobacillus lactis* Dorner in the absence of vitamin B_{12}, as indicated in the table. The purine requirements are less specific than that of thymidine, which cannot be replaced by thymine. Of the compounds tested, guanylic acid was the most effective of single purines or derivatives; however, mixtures of adenine and guanine or of hypoxanthine and guanine were practically as active.

Effects of Purines and Derivatives on Growth Responses to Thymidine and Vitamin B_{12}

Test organism, *Lactobacillus lactis* Dorner,* incubated 28 hours at 37–38°.

	Galvanometer reading†					
Constant supplement	Guanylic acid, 100 γ per 10 cc.	Thymidine, 10 γ per 10 cc.	Guanine and hypoxanthine, 100 γ each per 10 cc.	Thymidine, 10 γ per 10 cc.	Adenine and guanine, 100 γ each per 10 cc.	None
Variable supplement, γ each per 10 cc.	Thymidine	Guanylic acid	Thymidine	Guanine and hypoxanthine	Liver concentrate	Liver concentrate
0	11	15	10	15	14	9
1	28		27		24	
2	41		36		33	29
3		21		24	41	31
5	62		50		51	42
10	65	40	57	37	59	52
20						59
30		53		48		
100		66		57		

* In a previously described medium (foot-note 1) from which purines were omitted unless otherwise noted.

† A measure of culture turbidity; distilled water reads 0, an opaque object 100.

---

[1] Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Chem. Soc.*, **70**, 2614 (1948).

[2] Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, **175**, 475 (1948).

Three different concentrates of vitamin B_{12}[3] prepared by widely different processes replaced both purines (or derivatives) and thymidine in the nutrition of the organism; however, purines (or derivatives) had a slight sparing action which was never more than 2-fold. These results indicate that vitamin B_{12} is involved in the biosynthesis of purines (or derivatives) as well as thymidine but do not preclude the possibility that these substances in turn are also involved in the biosynthesis of vitamin B_{12}.

Dr. L. D. Wright has made available to us an organism, *Lactobacillus leichmannii* (ATCC 4797), which was used for assay during the isolation of the animal protein factor. In the nutrition of this organism, the animal protein factor can be replaced by thymidine.[4] In addition to this requirement, we have found that this organism requires folic acid for growth in a previously described medium containing purines.[1] The requirement for folic acid is replaced by thymine after a lag phase. Thymidine in the presence of folic acid adequately replaces the animal protein factor and also slowly replaces both folic acid and the factor after a lag phase. Hence, independent functions are indicated for folic acid and the animal protein factor, which presumably is identical at least functionally with vitamin B_{12}, in the biosynthesis of thymine and thymidine.

Biochemical Institute and the Department of Chemistry, The University of Texas, and the Clayton Foundation for Research
Austin

WILLIAM SHIVE
JOANNE MACOW RAVEL
W. M. HARDING

Received for publication, September 3, 1948

---

[3] Rickes, E. L., *et al.*, *Science*, **107**, 396. Shorb, *Science*, **107**, 397 (1948).
[4] Wright, L. D., private communication.

HIGHLY VISCOUS SODIUM HYALURONATE*

Sirs:

Two important properties have distinguished sodium hyaluronate as isolated heretofore from that occurring in the natural state as in normal synovial fluid or in aqueous extracts of umbilical cord. It possessed only a fraction of the viscosity of the native fluids and gave no mucin clot with acidified serum. The proposed explanations of this difference were either (1) that the substance had become oxidatively degraded during isolation[1] or (2) that secondary valences had broken down during the process of isolation.[2] The first explanation did not appear reasonable, since the mucopolysaccharide isolated in an atmosphere of nitrogen still possessed a relatively low viscosity.[3] In the experience of this laboratory, the isolated polysaccharide was quite stable in air at pH 6 to 7 and in fact was always isolated by vigorous stirring in air for many hours. The existence of secondary valences in the native fluids, on the other hand, was not proved by experiment.

By extraction of homogenized human umbilical cords with 2 per cent phenol, clotting the mixture after dilution with dilute acetic acid, removal of protein, and avoidance of a pH greater than 7.5, we have isolated preparations of sodium hyaluronate which in 0.3 per cent solution in buffered sodium chloride at pH 6 have relative viscosities as high as 32 compared to those of older preparations of 3 to 4 in the same solvents. On addition of diluted serum and acidification, the new preparations give a typical mucin clot. Half viscosity was obtained with the new preparations as substrates of testicular hyaluronidase with one-tenth to one-twentieth of a turbidity reducing unit, while, with older preparations of the polysaccharide, 3 to 4 units of the enzyme were required to reach half viscosity.[4]

The highly viscous preparations of hyaluronate under standard conditions precipitate 15 to 25 per cent less serum protein than older preparations, as measured turbidimetrically. They show, furthermore, a deficit of ash of about 20 per cent as calculated either from the uronic acid or hexosamine values. Suspension of the dry powdered sodium hyaluronate in

---

* This work was supported by grants from the Josiah Macy, Jr., Foundation and the Helen Hay Whitney Foundation.

[1] Blix, G., and Snellman, O., *Ark. Kemi, Mineral. o. Geol.*, **19 A**, 32 (1945).

[2] Meyer, K., *Physiol. Rev.*, **27**, 335 (1947).

[3] Skanse, B., and Sundblad, L., *Acta physiol. Scand.*, **6**, 37 (1943).

[4] In contrast to a recently published method these preparations represent the total extracted polysaccharide of the cord and are not fractionated into viscous and non-viscous fractions (Hadidian, Z., and Pirie, N. W., *Biochem. J.*, **42**, 2 (1948)).

dry ammoniacal methanol for 3 weeks resulted in an increase in nitrogen of 0.52 per cent, (from 2.72 to 3.24 per cent), or about 20 per cent. Only half of this additional nitrogen was ammonia nitrogen, while the rest was firmly bound, presumably in amide linkage.

The experimental findings appear to be explained by the assumption of the presence of acid anhydrides which bridged the glucosidic polymer chains to giant molecules. These anhydride bridges, which occur to the extent of about 20 per cent of the total glucuronic acid molecules present, presumably are opened during the isolation by the older procedure.

It is possible that some of the biological reactions such as some spreading effects and the mucin clot prevention test[5] are due to the hydrolysis of these labile oxygen bonds. Whether similar linkages occur in other natural high molecular compounds of great lability, such as some proteins and desoxyribonucleic acids, remains to be seen.

We wish to thank Miss Hannah Weinshelbaum and Miss Anita Steinberg for their assistance.

Departments of Ophthalmology and Medicine, College of Physicians and Surgeons, Columbia University, and the Edward Daniels Faulkner Arthritis Clinic, Presbyterian Hospital
New York

KARL MEYER

Received for publication, September 17, 1948

---

[5] McClean, D., Rogers, H. J., and Williams, B. W., *Lancet*, **1**, 355 (1943).

THE CONVERSION OF FORMATE AND GLYCINE TO SERINE AND GLYCOGEN IN THE INTACT RAT*

Sirs:

Glycine is generally agreed to be glycogenic. This net deposition of glycogen could be accounted for by the pathway via serine to pyruvate shown in Scheme I.

$$\mathrm{CH_2NH_2COOH + HCOOH \rightarrow CH_2OHCHNH_2COOH} \\ \rightarrow \mathrm{CH_3COCOOH} \rightarrow \text{glycogen} \qquad \text{(I)}$$

Evidence has been reported for the *in vitro* conversion of glycine to serine[1] and serine to pyruvate.[2] However, in the intact animal, the evidence was to the contrary.[3]

We have investigated this mechanism by degrading liver serine and glycogen isolated after the simultaneous administration of glycine labeled with C^{13} in the carboxyl group and formate containing C^{14}. According to Scheme I, the serine carboxyl group is derived from the corresponding group of glycine and should contain excess C^{13}, while the β-carbon atom, which is formed from formate, should be labeled with C^{14}. The glycogen should contain C^{13} in the 3,4 positions and C^{14} in all carbons, with the highest concentration in the 1,6 and lowest in the 3,4 positions. C^{14} in the 3,4 positions would result from fixation of $C^{14}O_2$ formed from the formate, while the reversible transformation of pyruvate into a symmetrical 4-carbon dicarboxylic acid would introduce the C^{14} of the β-carbon into the α position of pyruvate and the 2,5 positions of the glycogen.

Four fasted rats weighing a total of 571 gm. were given 5 mM of glycine by stomach tube and 0.125 mM of formate intraperitoneally per 100 gm. The latter dose was repeated at the end of the 5th hour. After 14 hours the animals were sacrificed. Glycogen was extracted from the livers with trichloroacetic acid and degraded by the procedure of Wood *et al.*[4] Serine was isolated from the neutral amino acid fraction of the liver hydrolysate as the *p*-hydroxyazobenzene sulfonate, m.p. 208–211° (decomposition) and degraded (Scheme II).

---

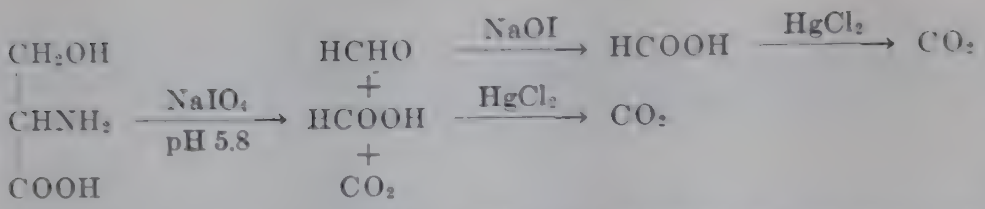
* Aided by a grant from the American Cancer Society, recommended by the Committee on Growth of the National Research Council, and by support of the Elisabeth Severance Prentiss Foundation.

[1] Winnick, T., Moring-Claesson, I., and Greenberg, D. M., *J. Biol. Chem.*, **175**, 127 (1948).

[2] Binkley, F., *J. Biol. Chem.*, **150**, 261 (1943). Chargaff, E., and Sprinson, D. B., *J. Biol. Chem.*, **151**, 273 (1943).

[3] Greenberg, D. M., and Winnick, T., *J. Biol. Chem.*, **173**, 199 (1948).

[4] Wood, H. G., Lifson, N., and Lorber, V., *J. Biol. Chem.*, **159**, 475 (1945).



$$\begin{array}{lcl} \mathrm{CH_2OH} & & \mathrm{HCHO} \xrightarrow{\mathrm{NaOI}} \mathrm{HCOOH} \xrightarrow{\mathrm{HgCl_2}} \mathrm{CO_2} \\ | & & + \\ \mathrm{CHNH_2} & \xrightarrow[\mathrm{pH\ 5.8}]{\mathrm{NaIO_4}} & \mathrm{HCOOH} \xrightarrow{\mathrm{HgCl_2}} \mathrm{CO_2} \\ | & & + \\ \mathrm{COOH} & & \mathrm{CO_2} \end{array}$$

(II)

The results of the carbon analyses, shown in the table, are in accord with Scheme I. Serine and glycogen contained considerable C^{13} and C^{14}. In serine, all of the C^{13} was located in the carboxyl carbon (Column 2), while most of the C^{14} was in the β position (Column 4). In glycogen the C^{13} was restricted to the 3,4 carbons (Column 2), while the C^{14} was distributed throughout the molecule with the highest concentration in the 1,6 positions. The absence of C^{13} from the β position of serine proves that this carbon does not arise from formate via CO_2 fixation.

Compound	Total C (1)		COOH or 3,4* (2)		α or 2,5* (3)		β or 1,6* (4)	
	C^{13}†	C^{14}‡	C^{13}†	C^{14}‡	C^{13}†	C^{14}‡	C^{13}†	C^{14}‡
Serine		133	0.78	4	0.00	20	0.00	377
Glycogen	0.27	343	0.81	212	0.00	321	0.00	512

The original formate and glycine carboxyl carbon contained 1.35 × 10^5 counts per minute per mg. of C and 9.98 atom per cent excess C^{13} respectively. 48 per cent of the formate C^{14} was recovered in the respiratory CO_2.

* Positions of the glucose unit of the glycogen.

† Atom per cent excess.

‡ Counts per minute per mg. of C.

This experiment demonstrates the likelihood of a pathway for the conversion of glycine and formate to glycogen via serine and pyruvate.

Department of Biochemistry
School of Medicine
Western Reserve University
Cleveland

WARWICK SAKAMI

Received for publication, September 20, 1948

PANCREATIC CARBOXYPEPTIDASE; A METAL PROTEIN*

Sirs:

It has been demonstrated that many exopeptidases are metal proteins as judged by the activation produced by specific metals, by inhibition studies with metal poisons, and by the slow reactivation by metal ions of the partially purified proteins (time reactions).[1] The crystalline carboxypeptidase[2] of bovine pancreas has appeared to be an exception to this, but it has not yet been studied from this viewpoint. We now wish to report that 5 times crystallized carboxypeptidase is inhibited by typical metal poisons. It is apparent from the table that the enzymatic action is completely blocked by 0.008 M sodium sulfide, and is strongly and progressively inhibited (approximately 80 per cent) by 0.008 M sodium cyanide. Sodium azide (0.008 M) has little or no effect on the enzyme.

Effect of Metal Inhibitors on Carboxypeptidase

The tests were performed at 25° with carbobenzoxyglycyl-L-phenylalanine (0.05 M) as the substrate in the presence of 0.067 M phosphate buffer at pH 7.4. Neutralized solutions of the inhibitors were mixed with the enzyme, allowed to stand for 5 minutes, and then added to the buffered substrate solution. Each test solution contained about 0.7 γ of protein N per cc. K is the first order velocity constant.

Inhibitor	Time	Hydrolysis	$K \times 10^3$	Inhibition
	hrs.	*per cent*		*per cent*
None	0.5	33	5.8	
	1.0	53	5.5	
	1.75	70	5.1	
0.008 M sulfide	26	1		100
0.008 " cyanide	1.0	22	1.8	80
	2.0	25	1.0	
	5.5	40	0.7	
0.008 " azide	1.0	52	5.2	0
	1.75	71	5.1	

The inhibition of this enzyme by these typical metal poisons suggests that carboxypeptidase is a metal protein like other exopeptidases. However, while most of the exopeptidases are exceedingly labile and lose their metal on prolonged dialysis or after mild purification procedures, the metal in carboxypeptidase must be much more firmly bound to the protein. Examination of the residual dry ash of our enzyme preparation in the arc of a sensitive spectrograph[3] has shown the presence of significant amounts of magnesium, and traces of iron and copper. Zinc, manganese, and cobalt, elements which are concerned in the activity of other peptidases, could not be detected. Moreover, no barium or lithium, which is used in the preparation and recrystallization[4] of the enzyme, could be found. The present evidence points towards magnesium as being the metal concerned in carboxypeptidase activity.

---

* This investigation was aided by a grant from the United States Public Health Service.

[1] Johnson, M. J., and Berger, J., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **2**, 69 (1942). Smith, E. L., and Bergmann, M., *J. Biol. Chem.*, **138**, 789 (1941); **153**, 627 (1944). Smith, E. L., *J. Biol. Chem.*, **163**, 15 (1946); **173**, 553, 571 (1948); **176**, 9, 21 (1948).

[2] Anson, M. L., *J. Gen. Physiol.*, **20**, 663 (1937).

Laboratory for the Study of Hereditary and Metabolic Disorders, and the Departments of Biochemistry and Medicine
University of Utah School of Medicine
Salt Lake City

EMIL L. SMITH
H. THEO HANSON[5]

Received for publication, October 2, 1948

---

[3] We are indebted to Professor H. R. Bradford of the College of Mines and Mineral Industries for this determination.

[4] Neurath, H., Elkins, E., and Kaufman, S., *J. Biol. Chem.*, **170**, 221 (1947).

[5] Postdoctorate Fellow of the United States Public Health Service.

CORRECTIONS

On page 82, line 28, Vol. 175, No. 1, August, 1948, read L-α-*GPC* for L-α-*GPA*.

On page 380, line 3 of "Integration," bottom of Table V, Vol. 176, No. 1, October, 1948, read *Sum of Fractions 37–40 and 45–47* for *Sum of Fractions 37–42 and 46–47*.

BIOCHEMICAL STUDIES ON THE WHOLE AND FRACTIONATED THYMUS OF RATS INJECTED WITH β-CHLOROETHYL VESICANTS*

By ALFRED CHANUTIN and STEPHAN LUDEWIG

(*From the Biochemical Laboratory, University of Virginia, Charlottesville*)

(Received for publication, June 9, 1948)

The sulfur and nitrogen mustards have a selective destructive action on lymphoid tissue (1–3). Assessment of the cellular damage caused by these compounds is based chiefly on histologic evidence. The thymus shrinks in volume owing to the destruction of lymphocytes, inhibition of mitosis, and the migration of lymphocytes into the blood stream (3). The present investigation is concerned with (*a*) the changes produced in the cholesterol, phospholipide, and nucleic acid of the whole thymus and (*b*) changes in the total lipide, cholesterol, and phospholipide of the stroma, lymphocyte nuclei, and saline-soluble proteins of the thymus after injection of β-chloroethyl vesicants.

Methods

Inbred male rats of Wistar stock, about 70 days old, were used as experimental animals. The agents and doses (LD_{100}) used were as follows: (*a*) bis(β-chloroethyl)sulfide (H), 0.4 mg. per kilo; (*b*) ethylbis(β-chloroethyl)amine hydrochloride (HN1), 0.4 mg. per kilo; (*c*) methylbis(β-chloroethyl)amine hydrochloride (HN2), 1.2 mg. per kilo; (*d*) tris(β-chloroethyl)amine hydrochloride (HN3), 0.6 mg. per kilo. The nitrogen mustards were dissolved in saline and H was dissolved in thiodiglycol immediately before injection. The agents, dissolved in 0.2 to 0.3 ml. of solvent, were injected into the exposed jugular vein of the animal anesthetized with sodium pentobarbital. Control animals were injected with saline. All rats were fasted after injection. At varying intervals after injection the rats were exsanguinated under anesthesia and the thymus was removed immediately, weighed, and prepared for analysis.

Methods for analyzing the cholesterol, phospholipide, and nucleic acid in the intact thymus are outlined. The total cholesterol was determined after the thymus had been dried to constant weight *in vacuo* over P_2O_5. The dried tissue was cut into small pieces with sharp scissors, transferred to an extraction thimble, and extracted under a reflux for 3 hours with 20 ml. of an absolute alcohol-acetone mixture (1:1). The extract was filtered and brought to 25 ml. with alcohol-acetone mixture. Aliquots were analyzed for total cholesterol by the Schoenheimer-Sperry procedure as modified by Sperry and Brand (4). Phospholipide phosphorus was determined in the individual fresh thymus of another group of rats. This tissue was homogenized with 20 ml. of an absolute alcohol-acetone (1:1) mixture and extracted under a reflux for 4 hours. Experiments were done to show that phospholipides were completely extracted by this procedure. The extract was brought to 25 ml. and aliquots were evaporated close to dryness on a hot-plate and the residue was immediately extracted with petroleum ether and the phosphorus was analyzed by the method of King (5) as modified by Allen (6). In a separate group of animals nucleic acid was determined spectrophotometrically by a modification of the method of Schneider (7). The whole thymus of each experimental rat was homogenized with the aid of 2 ml. of ice-cold distilled water and diluted to 10 ml. 2 ml. of the well mixed homogenate were transferred to a centrifuge tube, 4 ml. of 7.5 per cent trichloroacetic acid were added, and the mixture was heated at 90° for 15 minutes. After cooling, the tube was centrifuged and the supernatant was diluted 10 to 100 times with water, depending on the original thymus weight. Since the maximum absorption of nucleic acid dissolved in dilute trichloroacetic acid was at 260 mμ, all readings were made at this wavelength in a Beckman spectrophotometer. The nucleate content of a thymus extract was determined with the aid of a standard curve prepared with known amounts of calf thymus nucleate treated in the same manner as the unknowns.

---

* The work described in this paper was done under contract between the Medical Division, Chemical Corps, United States Army, and the University of Virginia. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

Fractionation procedures were developed in this laboratory (8) to obtain stroma, lymphocyte nuclei, and saline-soluble proteins of the thymus. In order to obtain sufficient thymus (7 to 10 gm.) to yield adequate amounts of these three fractions in each group, it was necessary to sacrifice thirty to forty control rats, and 90 to 100 HX3-injected animals. The fractions were dried to constant weight *in vacuo* over P_2O_5. The dried materials, which were ground to a powder, were first extracted with absolute ethanol overnight under a reflux, followed by a 6 hour extraction with absolute ethanol-ether (1:1). These extracts were combined and brought to 100 ml. with the ethanol-ether mixture. Total cholesterol (4), total lipide carbon (9), and phospholipide phosphorus (5, 6) were determined on aliquots of this extract.

The following conversion factors were used: lipide P × 23.7 = phospholipide, phospholipide × 0.647 = phospholipide carbon, and cholesterol × 0.839 = cholesterol carbon. The combined cholesterol and phospholipide carbon values were subtracted from total lipide carbon to give the concentration of "neutral fat" carbon.

Results

Intact Whole Thymus

Cholesterol (*Table I*)—The data for the mean values of dry weights of the individual thymuses and the percentage concentrations and contents of cholesterol after intravenous injection of saline and β-chloroethyl vesicants are summarized.

TABLE I

Dry Weights and Cholesterol Concentrations and Contents of Thymus of Fasting Rats after Intravenous Injection of Saline and Mustards

Days after injection	Saline	HN1	HN2	HN3	H
	Mg. dry weight thymus per 100 gm. rat				
1	57 ± 3.7 (10)	39 ± 1.8* (10)	43 ± 1.4* (10)	45 ± 2.2* (10)	40 ± 1.6* (10)
2	46 ± 2.5 (10)	31 ± 1.9* (21)	43 ± 1.7* (9)	29 ± 2.1* (10)	32 ± 2.8* (10)
3	44 ± 2.4 (12)	31 ± 1.4* (12)	21 ± 1.7* (12)	24 ± 1.3* (10)	23 ± 2.1* (8)
4	40 ± 1.9 (18)	34 ± 2.6 (9)	23 ± 1.2* (10)	19 ± 1.9* (10)	23 ± 1.7* (7)
5	36 ± 2.2 (10)	25 ± 2.2* (11)		17 ± 2.0* (11)	27 ± 4.1 (8)
	Mg. cholesterol per 100 gm. dry thymus				
1	1001 ± 37.7	1145 ± 45.6	1140 ± 44.6	1259 ± 50.0*	1297 ± 29.9*
2	1187 ± 66.8	1181 ± 66.7	1941 ± 104.1*	1627 ± 144.3*	1660 ± 120.5*
3	1184 ± 38.3	1988 ± 148.4*	2535 ± 150.8*	2039 ± 125.0*	2292 ± 118.5*
4	1546 ± 55.6	1484 ± 94.0	2641 ± 203.0*	2816 ± 246.0*	2411 ± 375.0
5	1622 ± 59.7	1891 ± 250.4		4151 ± 151.7*	2191 ± 310.2
	Mg. cholesterol per 100 gm. rat				
1	0.56 ± 0.04	0.45 ± 0.03	0.49 ± 0.02	0.56 ± 0.03	0.52 ± 0.02
2	0.54 ± 0.01	0.54 ± 0.02	0.61 ± 0.03	0.44 ± 0.20	0.50 ± 0.03
3	0.52 ± 0.03	0.61 ± 0.03	0.53 ± 0.04	0.48 ± 0.03	0.52 ± 0.03
4	0.60 ± 0.02	0.49 ± 0.02*	0.60 ± 0.02	0.50 ± 0.02	0.47 ± 0.04*
5	0.58 ± 0.02	0.47 ± 0.03*		0.65 ± 0.05	0.53 ± 0.05

The figures in parentheses represent the number of animals in each group.

* Represents significant difference from saline control.

Fasting causes an appreciable decrease in the thymus weight. The nitrogen and sulfur mustards are responsible for significant decreases in weight when compared with the saline controls. The greatest decreases are seen in the HN3 group.

The percentage concentrations of the cholesterol in dried thymus increase markedly in both the control saline and mustard groups. The remarkably constant values for the cholesterol content per 100 gm. of rat indicate a close relationship between the loss in thymus weight and the increased percentage concentration of cholesterol.

Phospholipide (*Table II*)—The data for the phospholipide concentration and content of the thymus (fresh) after injection of saline and HN3 are shown. The number of animals in each group is small and the standard errors, which are omitted, are relatively large. Although the percentage concentrations of phospholipide are consistently greater in the HN3 groups, there is no significant statistical difference from the saline controls. The decrease in the phospholipide content is statistically significant and is associated with the resulting marked involution after HN3.

Nucleic Acid (*Table III*)—Fasting of the control animals does not affect the percentage concentration of nucleic acid but does cause an appreciable decrease in the content of nucleic acid. The most marked decreases in both concentration and content of nucleic acid are observed after HN2 and HN3. Although HN1 is the most toxic of these agents, it causes the smallest change.

TABLE II

Percentage Concentrations and Contents of Phospholipide of Thymus of Fasting Rats after Intravenous Injection of Saline and HN3

Days after injection	Mg. per 100 mg. rat thymus		Mg. per 100 gm. rat	
	Saline	HN3	Saline	HN3
2	1.39 (5)	1.62 (5)	2.8	1.6
3	1.35 (5)	1.65 (5)	2.5	1.1
4	1.49 (8)	1.84 (7)	1.8	0.9
5	1.26 (5)	1.34 (7)	1.6	1.0

The figures in parentheses represent the number of animals in each group.

Thymus Fractions

Percentage Distribution (*Fig. 1*)—The percentage distribution of stroma, lymphocyte nuclei, and the saline-soluble proteins or cytoplasmic constituents of dry thymus at various periods after fasting and after the injection of saline or HN3 is shown. From these data it is clear that the increase in the stroma fraction is roughly proportional to the decrease in lymphocytes; the cytoplasmic components are not appreciably changed by the experimental procedures. A 4 day period of fasting in the saline group is responsible for a decrease in lymphocytes from 65 to 20 per cent; the value on the 4th day for the HN3 group is 3 per cent.

TABLE III

Nucleic Acid Concentrations and Contents of Thymus of Fasting Rats after Intravenous Injection of Saline and Mustards

Days after injection	Saline	HN1	HN2	HN3
Mg. nucleic acid per 100 mg. thymus				
1	2.14 ± 0.06 (9)	2.18 ± 0.06 (15)	1.45 ± 0.11* (10)	2.04 ± 0.04 (10)
2	2.20 ± 0.03 (10)	1.87 ± 0.08* (16)	1.31 ± 0.08* (11)	1.59 ± 0.08* (10)
3	2.13 ± 0.12 (10)	1.77 ± 0.07* (13)	1.05 ± 0.07* (9)	1.48 ± 0.04* (10)
4	1.97 ± 0.21 (10)	1.96 ± 0.07 (10)	1.28 ± 0.04* (10)	1.09 ± 0.05* (10)
5	1.85 ± 0.17 (10)	1.66 ± 0.10 (10)	1.16 ± 0.12* (10)	1.19 ± 0.08* (14)
Mg. nucleic acid per 100 gm. rat				
1	4.10 ± 0.18	3.48 ± 0.20	2.10 ± 0.15*	2.69 ± 0.14*
2	4.07 ± 0.14	2.27 ± 0.16*	1.47 ± 0.09*	1.44 ± 0.15*
3	3.60 ± 0.25	1.88 ± 0.13*	0.84 ± 0.08*	0.94 ± 0.07*
4	2.33 ± 0.21	2.16 ± 0.27	0.99 ± 0.06*	0.56 ± 0.04*
5	2.05 ± 0.17	1.49 ± 0.34*	0.93 ± 0.11*	0.76 ± 0.08*

The figures in parentheses represent the number of animals in each group.
* Represents significant difference from saline control.

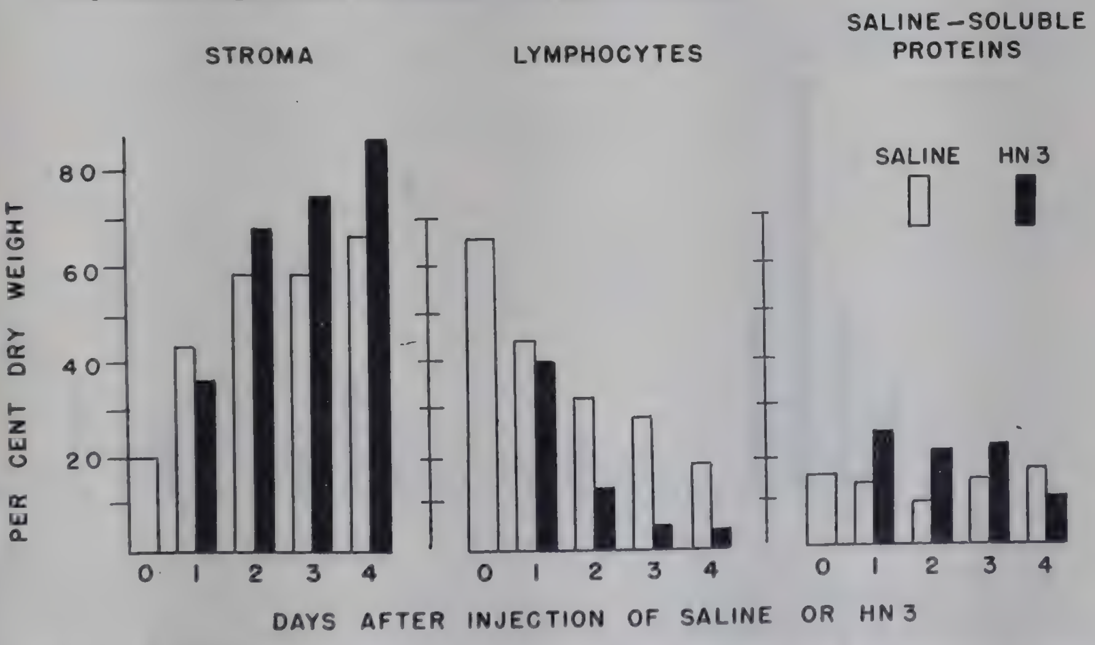


FIG. 1. Percentage distribution of stroma, lymphocytes, and saline-soluble proteins in the thymus of fasting rats injected with saline or HN3.

Lipide Analyses

Stroma (Fig. 2)—The concentration of total lipide carbon varies considerably in both groups owing to the neutral fat component. These vari-

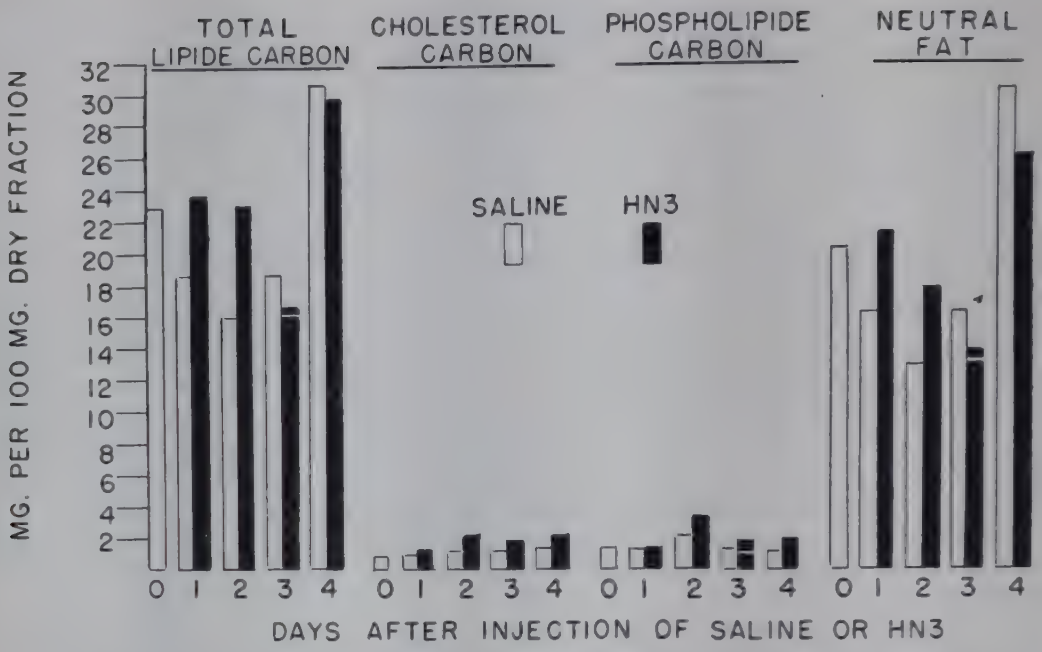


FIG. 2. Percentage concentration of total lipide, cholesterol, phospholipide, and neutral fat carbon in stroma of the thymus of fasting rats. Breaks in the graph represent two analyses on different samples.

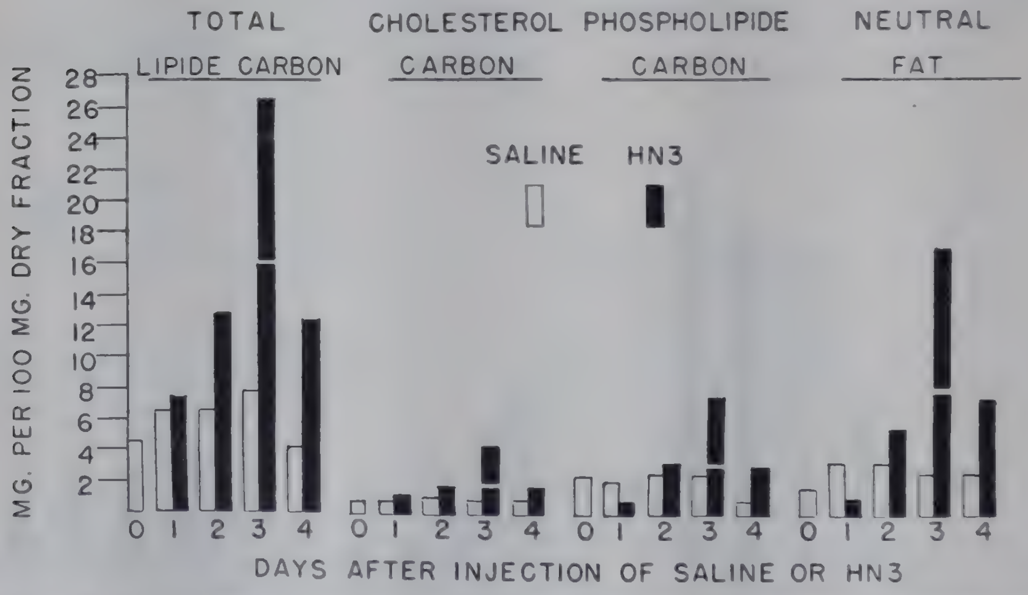


FIG. 3. Percentage concentration of total lipide, cholesterol, phospholipide and neutral fat carbon in lymphocyte nuclei of the thymus of fasting rats.

ations do not appear to be attributable to the effect of HN3. Both the cholesterol and phospholipide concentrations of the HN3 groups increase appreciably above most of the saline controls.

Lymphocyte Nuclei (Fig. 3)—Fasting in the saline-injected groups caused minor variations in the concentrations of the lipide components. After in-

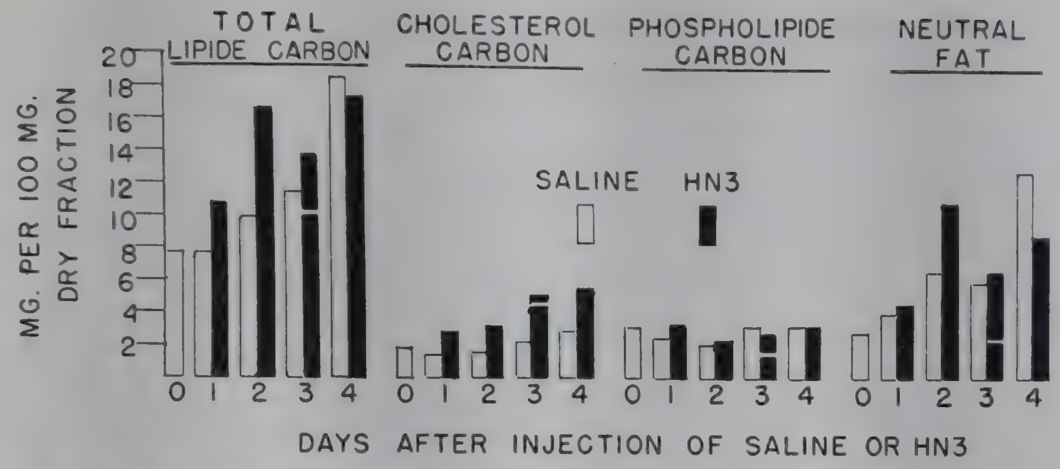


FIG. 4. Percentage concentration of total lipide, cholesterol, phospholipide, and neutral fat carbon in saline-soluble proteins of the thymus of fasting rats.

TABLE IV

Distribution of Cholesterol in Fractions of 100 Gm. of Dried Thymus of Fasting Rats after Saline and HN3 Injection

Days after injection		Saline		HN3	
		mg.	*per cent*	*mg.*	*per cent*
0	Stroma	193	18		
	Lymphocyte nuclei	535	50		
	Saline-soluble constituents	342	32		
		1070			
1	Stroma	410	41	396	23
	Lymphocyte nuclei	390	39	516	30
	Saline-soluble constituents	200	20	809	47
		1000		1721	
2	Stroma	744	60	1650	62
	Lymphocyte nuclei	310	25	265	10
	Saline-soluble constituents	186	15	742	28
		1240		2657	
3	Stroma	857	55	1600	56
	Lymphocyte nuclei	296	19	114	4
	Saline-soluble constituents	405	26	1140	40
		1558		2854	
4	Stroma	1090	59	2140	73
	Lymphocyte nuclei	185	10	88	3
	Saline-soluble constituents	574	31	701	24
		1849		2929	

jecting HN3, the total lipide concentration increases markedly on the 2nd day and reaches a maximum on the 3rd day. These increases are chiefly due to the increases in neutral fat. The values for cholesterol and phospholipide are consistently greater than those for the saline groups.

Saline-Soluble Components (*Fig. 4*)—This fraction contains the saline-soluble proteins and suspended particulate material of the cytoplasm. Fasting for 4 days is responsible for an impressive increase in lipide carbon from 7.7 to 18.6 per cent, owing chiefly to increases in neutral fat carbon; in the HN3 groups appreciable increases are noted on the 1st and 2nd days. The cholesterol concentrations in the nitrogen mustard groups are consistently increased about 100 per cent above the values of the saline controls. The phospholipide concentrations are not appreciably affected.

Cholesterol Distribution (*Table IV*)—The concentration and distribution of cholesterol in the three thymus fractions of rats injected with saline and HN3 are shown. This analysis yields information showing the fractions responsible for the constancy of the cholesterol values seen in these involuted glands (Table I). In both the saline and HN3 groups the cholesterol concentration of the stroma fraction increases from 18 to a maximum of 73 per cent of the total thymus. Concurrently the cholesterol content of the lymphocyte nuclei decreases from 50 to as low as 3 per cent of the total. The changes in the cholesterol content of the cytoplasmic fraction, particularly in the HN3 groups, are not markedly changed. The sums of the cholesterol content of the three fractions per 100 gm. of thymus increase in both the saline and HN3 groups and approximate closely the results shown for the whole thymus in Table I.

DISCUSSION

Despite the pronounced involution of the thymus, due chiefly to the loss of lymphocytes, the percentage concentration of cholesterol increases and the cholesterol content of the gland remains constant. The cholesterol of the lymphocyte nuclei of the control thymus accounts for 50 per cent of the total cholesterol; after HN3 injection, the lymphocytes account for 3 per cent of the total cholesterol of the involuted thymus. It is obvious that the stroma and the cytoplasm must contain the additional cholesterol to compensate for the deficit incurred by lymphocyte loss. It is therefore postulated that the thymus is capable of manufacturing a cholesterol-rich lipoprotein. It has been shown that the electrophoretic patterns and the lipide content of the plasma fraction II+III of the rat are changed after the injection of HN3.[1] It seems possible that some of the increased content of lipoprotein of the plasma of injured animals originates in lymphoid tissue.

---

[1] Chanutin, A., and Gjessing, E. C., unpublished results.

According to Kindred[2] the nuclei of the rat thymus lymphocytes show no morphologic changes during fasting. On the 1st day after injecting HN3, the largest number of degenerated lymphocytes is observed and a large proportion of the nuclei of surviving lymphocytes is partially lobulated. On subsequent days the number of degenerated cells decreases progressively, practically all of the lymphocyte nuclei are irregular in shape, but the distribution and appearance of the chromatin are normal. No correlation is apparent between the cytologic observations and the changes in the total lipide concentration of the nuclei. The very marked and sudden increases in the neutral fat concentration of the nuclei on the 2nd, 3rd, and 4th days after HN3 indicate extensive chemical alterations. This may represent the type of lipide infiltration commonly seen in cells after many types of chemical injury. A study of the literature has failed to disclose information dealing with the quantitative aspects of lipides of nuclei before and after injury.

SUMMARY

The effect of the intravenous injection of saline and tris(β-chloroethyl)-amine (HN3) upon the total cholesterol, phospholipide, and nucleic acid concentration of the thymus of the fasting rat is presented. The percentage concentration of cholesterol increases but the cholesterol content of the involuted thymus remains constant. The phospholipide and nucleic acid contents decrease.

Data are presented for the changes in the total lipide, cholesterol, and phospholipide carbon concentrations of the stroma, lymphocyte nuclei, and cytoplasmic constituents of the thymus after injection with saline and HN3. The pronounced effect of nitrogen mustard on decreasing the number of lymphocytes is demonstrated. HN3 causes a marked increase in the neutral fat of the lymphocytes, which is suggestive of lipide infiltration. The cholesterol concentration of each of the three fractions is elevated after HN3. The phospholipide concentration is slightly elevated in the stroma and lymphocyte nuclei.

Lymphoid tissue appears capable of synthesizing a cholesterol-rich lipoprotein.

The authors are indebted to Mr. Curtis S. Floyd, Miss Florence L. Jones, and Miss Elizabeth A. Lentz for technical assistance.

[2] Kindred, J. E., personal communication.

BIBLIOGRAPHY

1. Gilman, A., and Philips, F. S., *Science*, **103,** 409 (1946).
2. Graef, I., Karnofsky, D. A., Joger, V. B., Krichesky, B., and Smith, H. W., *Am. J. Path.*, **24,** 1 (1948).
3. Kindred, J. E., *Arch. Path.*, **43,** 253 (1947).
4. Sperry, W. M., and Brand, F. C., *J. Biol. Chem.*, **150,** 315 (1943).
5. King, E. J., *Biochem., J.*, **26,** 292 (1932).
6. Allen, R. J. L., *Biochem. J.*, **34,** 858 (1940).
7. Schneider, W. C., *J. Biol. Chem.*, **161,** 293 (1945).
8. Gjessing, E. C., Warren, T. N., and Floyd, C. S., *J. Nat. Cancer Inst.*, **9,** 43 (1948).
9. Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, **136,** 509 (1940).

GLYCOLYSIS IN TUMOR HOMOGENATES*

By G. A. LePAGE

(*From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison*)

(Received for publication, July 2, 1948)

Recent reports (1, 2) have strengthened the concept that tumor metabolism involves a very active glycolysis of the phosphorylating type. Suitable media for the study of glycolysis in homogenates of brain have been devised by Racker and Krimsky (3, 4) and Utter *et al.* (5, 6). These media were used, with slight modifications, by Novikoff *et al.* (2) for measurement of glycolysis in tumor homogenates. If the assumption that tumor uses high energy phosphate bonds as a source of energy for synthetic reactions is correct, then one important prerequisite for the study of synthetic reactions in tumor homogenates is the development of a medium which is optimal for sustaining phosphate bond energy in these homogenates. The object of this paper is to present data concerning the development of such optimal media for glycolysis in homogenates of Flexner-Jobling carcinoma. The medium for optimal glycolysis was developed not only on the basis of the Q_{CO_2}, but also in terms of lactate production, as determined chemically, and in terms of the esterification of inorganic phosphate.

EXPERIMENTAL

Flexner-Jobling carcinoma transplants in Sprague-Dawley albino rats were used throughout.[1] These were taken for experiment at 8 to 12 days after subcutaneous transplantation, at which time they weighed 500 to 1000 mg. This material had the advantage that it was very reproducible and as free as possible from necrotic tissue or other tissue elements.

The tumors were rapidly excised from decapitated rats and placed in small beakers of isotonic KCl immersed in chopped ice. After all outer connective tissue had been trimmed off, the tumors were weighed and added to cold Potter-Elvehjem (7) homogenizer tubes and alkaline[2] isotonic KCl

---

* This work was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

[1] The author wishes to acknowledge the assistance of Mr. B. E. Kline, who provided the tumor transplants.

[2] After preliminary experiments which indicated very little difference between the results obtained with homogenates made in isotonic KCl and those made in water, with a slight superiority of the former, alkaline KCl was routinely used. This alkaline KCl was isotonic KCl to which had been added 8.0 ml. of 0.02 M $KHCO_3$ per

was added to make the final tissue suspension 10 per cent. The Warburg respirometer vessels, in which the reactions were carried out, were prepared before the animals were killed, with all reactants added except the homogenate, and the vessels immersed in chopped ice. The homogenates were kept cold and used immediately after they were made. The medium in each reaction vessel had a final volume of 3.0 ml. when the homogenate was added. In the side arm was placed 0.25 ml. of 65 per cent trichloroacetic acid. At the end of the desired incubation time this trichloroacetic acid was mixed in with the contents of the main reaction vessel to give a final concentration of 5 per cent, which was sufficient to precipitate the proteins.

The measurements of tumor glycolysis were routinely carried out under anaerobic conditions, in an atmosphere of 95 per cent N_2-5 per cent CO_2. Novikoff *et al.* (2) demonstrated that 95 per cent O_2 was inhibitory to glycolysis of tumor homogenates. However, mixtures of air containing 5 per cent CO_2 (19 per cent O_2) gave results identical with those obtained anaerobically. In accomplishing the change of atmosphere in the reaction vessels, the method of Burris (9) was used. This involves setting up a manifold to which both arms of each Warburg manometer are attached. The manifold is connected at one end with a water aspirator, at the other with the source of the gas mixture. The reaction vessels are rapidly taken from the ice bath and attached to their respective manometers. The system is evacuated to 60 to 70 mm. of Hg with the water aspirator and the system refilled with the gas mixture. This is repeated two to three times. Then the manometers are detached from the manifold and the reaction vessels immersed in the water bath at 38° for incubation. This technique has the advantage that it conserves gas mixtures and can be carried out very rapidly. The lapse of time from removing the first of some twelve flasks from the ice until the last is in the 38° bath is approximately 5 minutes, and as a result the flask contents are still cold until they are immersed in the 38° bath. A 5 minute equilibration is sufficient before respiration measurements are begun. The gases used were commercial tank carbon dioxide and a specially purified nitrogen (99.99 per cent N_2).

In evaluating components of the medium or other factors the routine measurements made were (*a*) measurement of carbon dioxide evolution from the bicarbonate buffer, which gives a general evaluation of rate of reaction, but is not specific because acid equivalents are changed by several reactions (glycolysis, dephosphorylations, hexokinase reaction, etc.); (*b*) measurement of net inorganic phosphorus esterification, which is a measure of glucose phosphorylation, since none is esterified when glucose is not added to the medium; (*c*) lactic acid production, which is a more direct measure

---

liter (8). The alkaline KCl results in a homogenate which at the time of use is at pH 7.0.

of the amount of glycolysis than is carbon dioxide evolution. In some cases pyruvic acid and phosphoglyceric acid analyses were made to check upon the validity of the lactic acid measurements. The analytical methods were those described by LePage and Umbreit (9). Pyruvic acid was determined by the method of Friedemann and Haugen (10).

In approaching the problem of obtaining an optimal medium for sustaining phosphate bond energy, which could also be easily evaluated, certain compromises were necessary, since not one but a number of enzymes take part in the over-all reaction. It was thought desirable to use fluoride and block the reactions at the stage of phosphoglyceric acid. This limits the adenosinetriphosphatase activity as well. It has the advantage to the evaluation of the phosphorylations that phosphorus esterified is accumulated as a stable intermediate, 3-phosphoglyceric acid. It is possible to use a high fluoride level and get the theoretical esterification (1 mole of P esterified per phosphoglyceric acid produced). However, this also affects the glycolytic rate. Glycolysis is most rapid without any fluoride present, but in this case the end-reaction results in return of the phosphorus to the medium in inorganic form. Since the reaction was blocked with fluoride, it was necessary to add pyruvic acid as hydrogen acceptor. Upon establishing the validity of this, lactic acid measurements were used instead of phosphoglyceric acid analyses. Pyruvic acid was so low in the medium that interference with determination of lactate was insignificant.

TABLE I

Variation in Tonicity of Medium

Tonicity (isotonic saline as 1.00)	Lactic acid production per flask	Net P esterified per flask
	micromoles	*micromoles*
0.65*	10.4	4.2
1.00	9.95	2.8
1.24	9.15	1.5

* This is the tonicity of the final medium decided upon as optimal.

The medium of Novikoff *et al.* (2) was used at the start, and the components varied one at a time to determine optima. When all optima had been determined in this way, the necessary changes were made and the experiments repeated with the new conditions. All results represent averages of duplicate flasks in each of at least two closely agreeing experiments at the final conditions.

Tonicity of Medium—If isotonic saline is assigned a tonicity of 1.0, the medium used by Utter *et al.* (5) had a tonicity of approximately 0.73. That of Novikoff *et al.* (2) had a tonicity of approximately 1.17. Data are provided in Table I regarding the effects upon lactic acid production and net phosphorus uptake, in a 40 minute incubation, of varying tonicity in our optimal medium by additions of KCl.

Initial pH of Medium—In the medium, optimal in other respects, pH was varied by varying the bicarbonate concentration. The results are illustrated in Fig. 1. The values used are the initial pH before glycolysis has begun. The pH was checked after the gassing technique by rapidly removing the flasks and inserting a glass electrode in the contents. The pH values found agreed well with those predicted from calculations of the Henderson-Hasselbalch equation (9). The data in Fig. 1 are for a 40 minute incubation, during which separate experiments showed that the pH drop was 0.1 to 0.2 unit. The higher pH probably favors higher adenosinetriphosphatase activity. At lower than optimal magnesium concentrations, the range of optimal pH is much narrower, phosphorus uptake dropping sooner as pH progresses above 7.4.

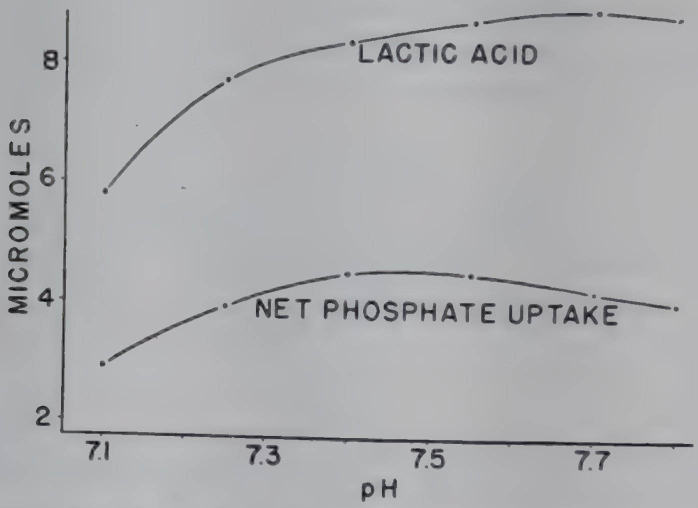


FIG. 1. Effect of initial pH of medium on lactic acid production and net phosphorus esterification per flask in a 40 minute incubation with 30 mg. of tissue.

Potassium versus Sodium—Boyer *et al.* (11) have discussed the necessity of potassium for phosphate transfers and demonstrated a potassium requirement for certain of these reactions. The substrates for our medium, the saline, bicarbonate, etc., were all potassium salts. Data provided in Table II demonstrate that potassium is necessary for optimal glycolysis, that it cannot be replaced completely by sodium without loss in efficiency, that the potassium level in the medium is sufficient, and that extra additions of potassium beyond 0.01 M do not alter the efficiency.

Substrates—Presumably the main substrate required for tumor glycolysis would be glucose. However, in operating a phosphorylating glycolysis, it is necessary first to phosphorylate the glucose. To permit this would make necessary the addition of relatively large amounts of adenosine triphosphate (ATP), which would lead to inefficiency in a homogenate system because of the active ATPase. Hence it is more practical to start the reaction by addition of a small amount of hexose diphosphate[3] (HDP). When no ATP or HDP was added to the reaction mixture, no lactic acid (less than 0.2 micromole) or phosphoglyceric acid was produced. When small amounts of HDP were added, the HDP was quantitatively converted, at a very rapid rate, to the equivalent of phosphoglyceric acid. When both glucose and HDP were added, a large increase in the phosphoglyceric acid production occurred above that theoretically possible from the HDP,[4] and inorganic phosphate was esterified. Large amounts of HDP decrease the net phosphorus uptake, probably by permitting more phosphatase activity and possibly by permitting a reaction rate exceeding the rate of the glucose-phosphorylating enzymes. Data concerning the effects of varying the HDP concentration in the otherwise optimal medium are presented in Fig. 2. A 40 minute incubation period was used. Similarly, effects of varying glucose concentration with HDP optimal are shown in Table III. The amount of glucose glycolyzed is independent of concentration, under these conditions, to very low levels. This is probably not true *in vivo* (12) as the HDP level is very low (1).

TABLE II

Potassium versus Sodium

Experimental conditions	Lactic acid production per flask	Net P esterified per flask
	micromoles	*micromoles*
All sodium salts	2.6	−0.3
Potassium, 0.01 M; all other salts sodium	10.4	4.0
" salts entirely (K approximately 0.07 M)	10.5	4.1

Pyruvate—Pyruvate is added to act as hydrogen acceptor. This is necessary because the normal reactions which would provide it are blocked by the fluoride. However, when fluoride is absent, the system still derives some benefit from addition of a catalytic amount of pyruvate to prime the reaction. Measurements concerning the effects of adding or omitting pyruvate are presented in Table IV. The data for no pyruvate addition with fluoride present permit calculation of the extent to which the fluoride is inhibiting at the phosphoglyceric acid stage. The inhibition with 0.01 M KF is indicated to be approximately 90 per cent.

---

[3] Hexose diphosphate used in this study was prepared from HDP obtained as barium salt from the Schwarz Laboratories, Inc., New York, by precipitation as the acid barium salt. Before it was used, the barium was removed with sulfuric acid and the HDP neutralized with potassium bicarbonate.

[4] Production of lactic acid in the absence of added glucose exceeds the theoretical amount obtainable from added HDP to a small extent. A corresponding amount of pyruvic acid disappears from the lactic-pyruvic sum, indicating the possibility that a dismutation of 2 molecules of pyruvic to 1 of lactic, 1 of acetic, and 1 of carbon dioxide occurs. Direct evidence that this is the case has not yet been obtained.

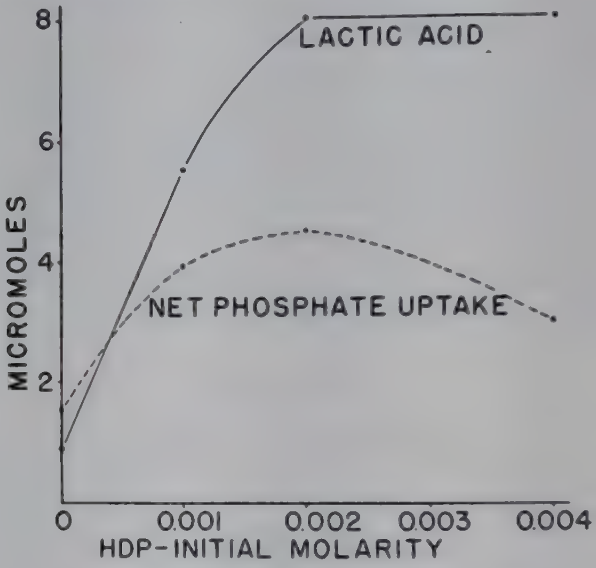


FIG. 2. Effects of varied HDP additions on micromoles of lactic acid produced and net phosphorus esterified per flask in a 40 minute incubation with 30 mg. of tissue.

TABLE III

Effect of Varied Glucose Concentration on Glycolysis

Glucose concentration	Lactic acid produced per flask	Net P uptake per flask
M	*micromoles*	*micromoles*
0	7.6	0
0.0013	10.2*	3.9
0.01	10.4	4.2
0.02	10.1	4.2

* This actually represents glycolysis of two-thirds of the added glucose.

Magnesium and Fluoride—In studies of these two ions, variations were made in both, since the concentrations being studied are such that the medium is close to or above saturation with magnesium fluoride. However, no indications were obtained that any precipitation was occurring. Table V illustrates the effect of variations in magnesium with fluoride optimal (0.01 M) and Table VI illustrates the effects of varied fluoride concentrations with magnesium constant (0.0066 M). The decision was made to use 0.01

M fluoride, despite higher phosphorylation efficiency with higher levels, because of the inhibiting effect on the glycolytic rate of such increases in fluoride.

TABLE IV

Effects of Added Pyruvate on Glycolysis in 40 Minute Incubation

Conditions	Lactic acid produced per flask	Net P uptake per flask
	micromoles	*micromoles*
Complete medium	9.2	4.0
Minus pyruvate	0.9	−0.5
" " minus fluoride	8.0	−4.4*
" fluoride + 0.1 optimal pyruvate	10.0	−3.2
" " + optimal pyruvate	11.0	−3.2

* The theoretically possible net P loss to the medium from the added HDP alone is 12 micromoles.

TABLE V

Effects of Varied Magnesium Concentration in 40 Minute Incubation

Magnesium concentration	Lactic acid produced per flask	Net P uptake per flask
M	*micromoles*	*micromoles*
0	7.60	−0.23
0.00165	8.85	0.55
0.0033	8.85	1.95
0.0066	8.75	3.5
0.0099	8.2	3.5
0.0133	7.9	3.5

TABLE VI

Effects of Varied Fluoride Concentrations on Glycolytic Rate During 40 Minute Incubation

Potassium fluoride concentration	Lactic acid produced per flask	Net P uptake per flask
M	*micromoles*	*micromoles*
0	10.5	−10.5
0.0066	8.45	1.6
0.010	8.75	3.5
0.020	6.75	4.5
0.030	5.85	4.7

Adenosine Triphosphate—The adenosine phosphates are known to be coenzymes or "carriers" in the mechanism of enzymatic phosphate transfer. Experiments with adenylic acid and ATP, the former prepared by the method of Kerr (13), the latter by the method of LePage (9), both in pure state, indicated that they were equivalent. A slightly higher phosphorus uptake is noted with adenylic acid. The effects of varying ATP concentration in the medium are illustrated in Fig. 3. Net phosphorus uptake tends to decrease at higher levels. This is undoubtedly due to increased ATPase activity at higher substrate levels.

Diphosphopyridine Nucleotide (*DPN*)—This compound is known to be required as coenzyme for hydrogen transfer in glycolysis. For use in our experiments it was prepared by the method of LePage (14) and further purified by solution in acid methanol and precipitation with ethyl acetate. This purification was employed to lower the content of adenylic acid (the chief impurity) in order to permit more quantitative definition of the ATP requirement. The preparations as used assayed 85 per cent DPN and contain less than 10 per cent of adenylic acid (without calculation for water of hydration). Fig. 4 illustrates the effects of varying additions of DPN.

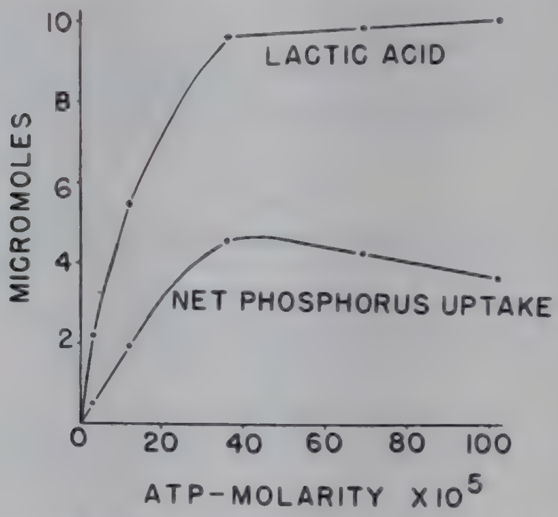


FIG. 3. Effects of varied ATP concentration on lactic acid production and net phosphorus esterification by 30 mg. of tissue in a 40 minute incubation.

Protective Factors—One compound cited as a protective factor for glycolytic enzymes is glutathione (15). Addition of glutathione to the medium had no effect upon duration or rate of glycolysis in our tumor homogenates.

Novikoff *et al.* (2) demonstrated that nicotinamide had a protective effect on the tumor homogenate system. Presumably this is due to its inhibitory effect on the nucleosidase breaking down DPN. Recently it has been shown that α-tocopherol phosphate inhibits breakdown of DPN in various rat tissues (16) and that a pyrophosphatase rather than a nucleosidase is responsible for DPN breakdown in kidney (17). It was therefore of interest to determine the effects of both nicotinamide and α-

tocopherol phosphate[5] on preservation of DPN in this tumor homogenate system. Data on lactic acid production and phosphorus uptake, in a 40 minute incubation, are given in Table VII. DPN breakdown in these experiments was markedly inhibited by nicotinamide, but was unaffected by α-tocopherol phosphate.

Inorganic Phosphate—Inorganic orthophosphate is obviously necessary in the phosphorylating glycolysis mechanism. A discussion of its possible rôle in regulation of rate was provided by Potter (18). It was of value in making accurate measurements of phosphate uptake to have the phosphate of the medium relatively low. It is necessary to reduce the phosphate of the medium to a very low level before it affects the glycolytic rate. Data to illustrate this are provided in Table VIII. The lowest initial concentration listed there represents no phosphate addition, and is that resulting from the phosphorus present in the added tissue.

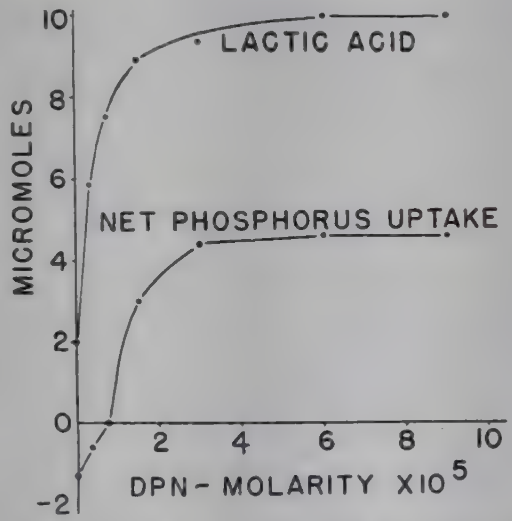


Fig. 4. Effects of varied DPN on lactic acid production and net phosphorus esterification by 30 mg. of tissue in a 40 minute incubation.

Homogenate—In all of the experiments described above, 30 mg., wet weight, of tumor were used per reaction vessel. This gives a suitable rate of reaction. Data provided in Fig. 5 for a 20 minute incubation illustrate that the lactic acid produced (phosphoglyceric) is proportional to the amount of tissue added. When incubation is carried beyond this time, the linear relationship fails because HDP becomes limiting. The phosphorylation of glucose with the phosphate bond energy gained from the triose phosphate dehydrogenase reaction is not 100 per cent efficient, owing to ATPase activity, and consequently the HDP concentration progressively

---

[5] The author wishes to thank Dr. Stanley Ames of Distillation Products, Inc., for a gift of α-tocopherol phosphate.

TABLE VII

Effects of Nicotinamide and α-Tocopherol Phosphate in 40 Minute Incubation

Conditions	Lactic acid production per flask	Net P uptake per flask
	micromoles	*micromoles*
Unprotected system	2.8	0.65
" " + α-tocopherol phosphate, 1 mg. per flask	3.15	0.68
Unprotected system + nicotinamide, 0.04 M	7.1	4.2

TABLE VIII

Effect of Varied Inorganic Phosphate Levels in 40 Minute Incubation

Initial phosphate concentration	Final phosphate concentration	Net P uptake per flask	Lactic acid produced per flask
$M \times 10^{-3}$	$M \times 10^{-3}$	*micromoles*	*micromoles*
0.25	0.12	0.37	3.2
0.37	0.11	0.82	3.4
0.53	0.14	1.1	3.6
0.80	0.14	1.9	4.6
1.34	0.32	3.0	8.1
2.7	1.2	4.2	9.0

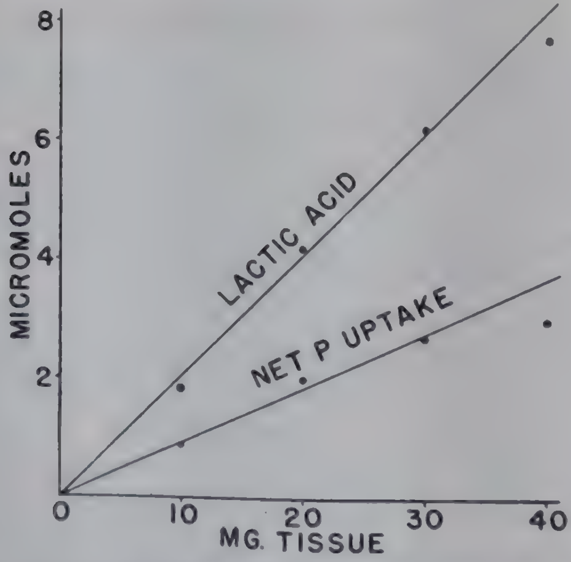


FIG. 5. Effects of varied additions of tissue on lactic acid production and net phosphorus esterification in a 20 minute incubation.

falls. During the first 20 minutes incubation, HDP has not become limiting, although more has been glycolyzed than was initially present (6.0 micromoles added).

The optimal medium finally selected had the following molar composition: potassium phosphate, 0.0024; potassium bicarbonate, 0.025; nicotinamide, 0.040; adenosine triphosphate,[6] 0.00033; diphosphopyridine nucleotide,[6] 0.00020; hexose diphosphate,[6] 0.0020; pyruvic acid,[6] 0.0050; magnesium chloride, 0.0066; potassium fluoride, 0.010; glucose, 0.010.

Rate of Reaction—The data in Fig. 6 illustrate the rate of reaction in typical experiments with optimal homogenate medium and 30 and 10 mg. of tissue. For some purposes it would be desirable to use only 10 mg. Nearly the same linearity of rate can be obtained with the higher tissue concentrations, however, if more HDP is added.

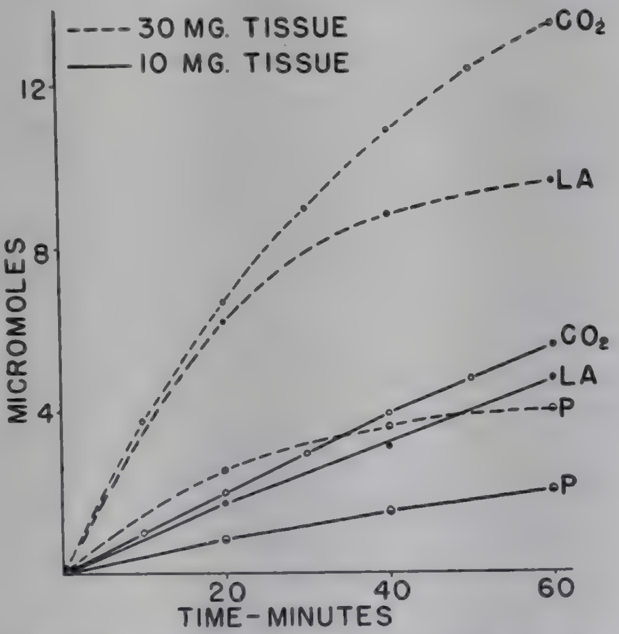


FIG. 6. Reaction rates in the optimal homogenate medium obtained with tw tissue levels. CO_2 = carbon dioxide evolved from the bicarbonate buffer per flask LA = lactic acid produced per flask; P = net phosphorus esterification per flask.

DISCUSSION

It was important to prove that high energy phosphorus in the form of ATP was present throughout the reaction period. Preliminary studies concerning this indicate that at least two-thirds of the ATP originally added is present as such after a 40 minute incubation. Further studies regarding the rates of transformation of the various phosphorylated intermediates will be made the subject of a later publication.

Some studies have been made concerning the glycolytic rates obtainable with homogenates of normal tissues. Of those examined, all appear to be able to glycolyze HDP very rapidly. Rat brain homogenate, in a medium similar to the one herein described, is able to glycolyze glucose and give large phosphorus uptakes. However, rat liver and kidney produce phosphoglyceric acid theoretically equivalent to the HDP added and do not glycolyze glucose or take up phosphorus in this medium. The study of conditions necessary to obtain phosphorylation of glucose in rat liver and kidney homogenates will be the subject of a later publication.

---

[6] Added as potassium salts.

SUMMARY

With Flexner-Jobling rat carcinoma transplants as the tissue source, a medium has been devised for glycolysis in tumor homogenates. This medium permits glycolysis of glucose, esterification of inorganic phosphate, and maintenance of phosphate bond energy.

BIBLIOGRAPHY

1. LePage, G. A., *Cancer Res.*, in press.
2. Novikoff, A. B., Potter, V. R., and LePage, G. A., *Cancer Res.*, in press.
3. Racker, E., and Krimsky, I., *J. Biol. Chem.*, **161**, 453 (1945).
4. Racker, E., and Krimsky, I., *J. Exp. Med.*, **84**, 191 (1946).
5. Utter, M. F., Reiner, J. M., and Wood, H. G., *J. Exp. Med.*, **82**, 217 (1945).
6. Utter, M. F., Wood, H. G., and Reiner, J. M., *J. Biol. Chem.*, **161**, 197 (1945).
7. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
8. Potter, V. R., LePage, G. A., and Klug, H. G., *J. Biol. Chem.*, **175**, 619 (1948).
9. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis (1945).
10. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943).
11. Boyer, P. D., Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, **146**, 673 (1942).
12. Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, **65**, 397 (1925).
13. Kerr, S. E., *J. Biol. Chem.*, **139**, 131 (1941).
14. LePage, G. A., *J. Biol. Chem.*, **168**, 623 (1947).
15. Rapkine, L., *Compt. rend. Acad.*, **207**, 301 (1938).
16. Spaulding, M. E., and Graham, W. D., *J. Biol. Chem.*, **170**, 711 (1947).
17. Kornberg, A., and Lindberg, O., *Federation Proc.*, **7**, 165 (1948).
18. Potter, V. R., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **4**, 201 (1944).

CENTRIFUGAL FRACTIONATION OF GLYCOLYTIC ENZYMES IN TISSUE HOMOGENATES*

By G. A. LePAGE and WALTER C. SCHNEIDER

(*From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison*)

(Received for publication, July 19, 1948)

Experiments on aerobic and anaerobic glycolysis in mammalian tissues have usually been conducted either with slices or with extracts. The latter have usually been made by breaking up the tissue and centrifuging and discarding some fraction of the particulate matter. Studies with slices have disadvantages in certain cases because of permeability factors. Results with extracts are frequently misleading because of variability in the amount of material discarded and lack of knowledge concerning the relation between the activity of the extract and that of the discarded portion. When reactions are studied for which information is not available as to intracellular distribution of the enzymes involved, it is advantageous to use whole homogenates. In this case permeability factors are largely ruled out and no part of the activity is discarded.

The complexities with regard to intracellular distribution of enzymes involved in glycolysis studies have been discussed by Meyerhof *et al.* (1–3). Recently a medium was devised for studies of anaerobic glycolysis with homogenates of Flexner-Jobling rat carcinoma, which enables these homogenates to maintain phosphate bond energy for reasonable periods of time and consequently permits studies of synthetic reactions with these homogenates (4). This medium was found to be approximately optimal for other tissues as well.

Centrifugal fractionations of tissue homogenates now permit separation of intracellular material into four well defined fractions (5): (*a*) a nuclear fraction, (*b*) the mitochondria or "large granule" fraction, (*c*) submicroscopic particle fraction, and (*d*) a supernatant fluid containing soluble enzymes. The object of this paper is to present the results of experiments in which homogenates were separated into these four fractions and recombined in the various possible combinations. Measurements were made of lactic acid production and net uptake of inorganic orthophosphate by the fractions.

---

* This work was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

EXPERIMENTAL

Since the centrifugal fractionation procedure requires some 5 hours, and the glycolytic measurements cannot be taken until fractionation is completed, it was first necessary to study the stability of the enzyme systems. Data presented in Table I indicate that little decline in activity occurs in 5 hours at 0°, but that considerable decline occurs in 24 hours. It was therefore possible to complete the fractionation and make the glycolytic measurements before any significant fraction of the activity was lost.

For the study of enzyme distribution in tumor cells, Flexner-Jobling carcinoma transplants[1] were made in Sprague-Dawley rats and the tumors taken 8 to 12 days later, when they were actively growing and free from necrosis. Liver is a very suitable normal tissue for this fractionation procedure. It is composed mainly of one type of cell and provides clear cut fractions. However, homogenates of liver tissue from intact, normal rats, though they glycolyze hexose diphosphate rapidly, do not appear to glycolyze glucose; they give inorganic phosphate output on our medium. Rabbit liver was found to be more suitable, and consequently it was used as an example of a normal, differentiated tissue. The rabbits used were New Zealand white of mixed sex, 6 to 8 weeks of age. These rabbits were fasted 24 hours before use to eliminate glycogen from the liver.

TABLE I

Stability of Glycolytic System in Homogenates Stored at 0°

Glycolysis obtained in 40 minutes with 30 mg. of tissue. The results are expressed in micromoles per flask.

Age of homogenate at 0°	Flexner-Jobling carcinoma		Rat liver		Rabbit liver	
	Lactic acid	Net P uptake	Lactic acid	Net P uptake	Lactic acid	Net P uptake
hrs.						
0	9.4	4.0	6.8	−2.8	6.4	0.6
5	9.0	3.6	6.2	−2.8	6.1	0.5
24	2.6	1.0	3.4	−3.0	3.0	−0.9

Fractionation—The animals were killed by decapitation and the tissues were rapidly removed to beakers containing ice-cold isotonic KCl and immersed in chopped ice, weighed, and homogenized in 9 volumes of isotonic (8.5 per cent) sucrose solution. The homogenates were fractionated exactly as previously described (5), with one exception. In the case of the tumor homogenates, the nuclear fraction was not washed because some of the nuclei failed to sediment when resuspended and centrifuged. Four fractions were obtained for each tissue: a nuclear fraction (N or N_W), a mitochondrial fraction (M_W), a submicroscopic particle fraction (P_W), and a supernatant fluid (S_2). The fractions were examined microscopically. The nuclear fraction contained large numbers of intact nuclei, some unbroken cells, and no free mitochondria. Intact nuclei or nuclear fragments were not visible in any of the other fractions. The nitrogen and nucleic acid contents of the fractions are given in Tables II and III. Each figure represents the average of three separate experiments. Nitrogen was de-

---

[1] The authors wish to acknowledge the assistance of Mr. B. E. Kline in preparing the tumor transplants.

TABLE II

Total Nitrogen and Nucleic Acid Content of Fractions Obtained from Homogenates of Flexner-Jobling Rat Carcinomas

Fraction	Nitrogen*		PNA phosphorus*			DNA phosphorus*		
	Total	A	Total	A	B	Total	A	B
	γ	*per cent*	γ	*per cent*	γ	γ	*per cent*	γ
Homogenate	1503	(100)†	55.2	(100)†	36.7	46.1	(100)†	30.6
N	559	37.2	20.9	37.8	37.4	39.4	85.4	70.7
M_W	124	8.3	5.8	10.5	46.7			
P_W	130	8.7	9.4	17.0	72.2			
S_2	759	50.5	17.8	32.2	23.4			
Recovery..	1572	104.7	53.9	97.5		39.4	85.4	

* Per 100 mg. of fresh tissue or its equivalent. Phosphorus calculated from pentose determinations (7). A, fraction of homogenate; B, per mg. of N.

† Figure assumed to be 100 per cent.

TABLE III

Total Nitrogen and Nucleic Acid Content of Fractions Obtained from Homogenates of Rabbit Liver

Fraction	Nitrogen*		PNA phosphorus*			DNA phosphorus*		
	Total	A	Total	A	B	Total	A	B
	γ	*per cent*	γ	*per cent*	γ	γ	*per cent*	γ
Homogenate	2580	(100)†	4.7	(100)†	18.1	22.4	(100)†	8.7
N_W	574	22.2	13.1	28.0	22.8	22.3	99.5	38.9
M_W	272	10.6	4.6	9.8	17.0			
P_W	373	14.5	15.2	32.5	40.8			
S_2	1260	48.9	14.8	31.6	11.8			
Recovery..	2479	96.2	47.7	101.9		22.3		

* Per 100 mg. of fresh liver or its equivalent. Phosphorus calculated from pentose determinations (7). A and B, see Table I.

† Figure assumed to be 100 per cent.

TABLE IV

Flexner-Jobling Carcinoma

Glycolysis obtained in 40 minutes with 30 mg. of tissue or fraction obtained therefrom.

Tissue fraction	Lactic acid produced per flask	Net P uptake per flask
	micromoles	*micromoles*
Homogenate	7.35	3.62
Nuclei (N)	1.31	1.23
Mitochondria (M_W)	0	−0.13
Microsomes (P_W)	0.05	−0.76
Supernatant fluid (S_2)	2.69	2.07
$N+M_W$	1.99	1.19
$N+P_W$	2.28	0.62
$N+S_2$	4.23	2.42
M_W+P_W	0.38	−0.46
M_W+S_2	4.25	2.78
P_W+S_2	4.85	2.15
$N+M_W+P_W+S_2$	7.28	3.33

TABLE V

Rabbit Liver

Glycolysis obtained in 40 minutes with 30 mg. of tissue or fraction obtained therefrom.

Tissue fraction	Lactic acid produced per flask	Net P uptake per flask
	micromoles	*micromoles*
Homogenate	6.26	0.23
Nuclei (N_W)	0.79	0.26
Mitochondria (M_W)	0	0.05
Microsomes (P_W)	0.17	−0.31
Supernatant fluid (S_2)	3.30	0.42
N_W+M_W	0.84	0.49
N_W+P_W	1.74	−0.14
N_W+S_2	4.76	0.41
M_W+P_W	0.55	−0.15
M_W+S_2	4.30	0.07
P_W+S_2	5.94	0.27
$N_W+M_W+P_W+S_2$	6.55	0.20

termined by a micro-Kjeldahl procedure (6) and nucleic acids by colorimetric reactions (7).

Glycolytic Measurements—These were made in Warburg respirometer flasks at 38° under anaerobic conditions in an atmosphere of 95 per cent nitrogen-5 per cent carbon dioxide. The gassing of flasks and other manipulations were as described earlier (4). To each flask the following additions were made: 0.30 ml. of 0.024 M potassium phosphate (pH 7.4), 0.15 ml. of 0.5 M potassium bicarbonate, 0.30 ml. of 0.4 M nicotinamide, 0.15 ml. of 0.15 M potassium pyruvate, 0.10 ml. of 0.01 M adenosine triphosphate potassium salt, 0.20 ml. of 0.003 M diphosphopyridine nucleotide potassium salt, 0.20 ml. of 0.1 M magnesium chloride, 0.10 ml. of 0.3 M glucose, 0.15 ml. of 0.04 M hexose diphosphate potassium salt, 0.15 ml. of 0.2 M potassium fluoride, 0.30 ml. of water, isotonic sucrose (8.5 per cent) or tissue fraction in isotonic sucrose to make a total of 3.0 ml. Homogenate equivalent to 30 mg. of tissue (0.30 ml.) was used for each flask. The washed particle fractions were resuspended in isotonic sucrose to one-third the volume of the homogenate from which they were obtained and a corresponding amount used (0.10 ml. per flask). The supernatant fluid had necessarily a larger volume and correspondingly more of it was used per flask (0.60 ml.). The glycolysis measurements were made as soon as the fractionation was completed (5 hours). Carbon dioxide output was measured at 38° for 40 minutes; then 0.25 ml. of 65 per cent trichloroacetic acid was tipped in from the side arm of each flask to stop the reaction. The flask contents were analyzed for lactic acid and inorganic phosphorus by methods previously described (6). Data for carbon dioxide evolution are omitted in favor of direct analyses for lactic acid, since the latter are more specific.

Table IV gives the results of glycolysis measurements with Flexner-Jobling carcinoma homogenates, fractions, and recombinations. Table V gives the results obtained similarly for rabbit liver. Each figure in the tables represents the average of results from three separate experiments. Variations from one experiment to another were small.

DISCUSSION

The data for nucleic acid and nitrogen (Tables II and III) merit some comparison with the results obtained with other tissues (5, 8). The distribution of nitrogen in the fractions obtained from homogenates of Flexner-Jobling carcinoma was similar to that observed with liver tumors (8). The largest amounts of nitrogen were found in the nuclear fraction and supernatant, while the mitochondria and submicroscopic particle fractions contained very small amounts of nitrogen. Pentose nucleic acid (PNA) was present in all fractions but was more concentrated in the mitochondrial and submicroscopic particle fractions than in the homogenate. The reason for the poor recovery of desoxypentose nucleic acid (DNA) in the nuclear fraction of the Flexner-Jobling carcinoma (85.4 per cent) is not known, since microscopic examination of the fractions indicated that nuclear material was present only in the nuclear fraction. A comparison of the results obtained with rabbit liver (Table III) with those previously reported for rat liver (5) shows that the most striking difference between the two tissues was the greatly decreased amounts of nitrogen recovered in the mitochondrial and submicroscopic particle fractions of the former. As in the case of rat liver, PNA was more concentrated only in the submicroscopic particle fraction and the entire DNA of the rabbit liver homogenate was recovered in the nuclear fraction.

The glycolysis data can be examined with regard to which fractions of the cell are able to glycolyze hexose diphosphate (*i.e.*, form lactic acid) and which are able to give phosphorus uptake. The interpretation is admittedly complicated by the possibility that lactate formation may be limited by lack of any of the enzymes concerned with the phosphate acceptor-transmitter system. When these are absent from a fraction, its glycolysis might cease owing to lack of a phosphate acceptor. It is known that adenosinetriphosphatase tends to be associated with the particulate matter (3, 8).

The results for tumor and normal liver are quite similar with regard to distribution of glycolytic enzymes. Liver does not give the strong phosphorylation reaction that is obtained with tumor under these conditions. It appears that the main glycolytic activity is in the soluble fraction (S_2). However, it is not possible to get a rate approaching that of the homogenate without the addition to the soluble enzymes of one of the particulate fractions. Since the phosphorus uptakes by the soluble fraction are quite efficient, in relation to the amount of glycolysis, without the addition of particulate fractions, it does not seem likely that the hexokinase is lacking from the soluble fraction. It therefore appears that the particulate matter is needed mainly to add adenosinetriphosphatase in order that phosphate acceptor may be made more rapidly available. It is obvious that no fraction or pair of fractions can give the full activity of the whole homogenate, though when all fractions are recombined, the original activity is achieved. In the tumors, no single fraction gave as high an activity per unit of protein nitrogen as the original homogenate.

Some glycolytic activity is shown by the nuclear fractions. This is probably due to the presence of some whole cells, and in the case of the tumor to the presence of impurities from the other fractions, since the tumor nuclei were not washed.

SUMMARY

Homogenates of Flexner-Jobling rat carcinoma and rabbit liver were separated centrifugally to give four definite fractions: (*a*) a nuclear fraction, (*b*) a mitochondrial fraction, (*c*) a submicroscopic particle fraction, (*d*) a

"soluble' fraction. Measurements were made of lactic acid production and net phosphorus uptake in an optimal medium under anaerobic conditions. The glycolytic enzymes appear to be in the "soluble" fraction, though addition of any of the particulate fractions markedly increases the rate. No single fraction or pair of fractions is able to reach the activity of the original homogenate, though this is achieved when all fractions are recombined. It is concluded that in any study of glycolytic enzmyes no fraction of the cell should be used without making a study of the relationship between it and the total cell contents.

BIBLIOGRAPHY

1. Meyerhof, O., and Geliazkowa, N., *Arch. Biochem.*, **12**, 405 (1947).
2. Meyerhof, O., *Arch. Biochem.*, **13**, 485 (1947).
3. Meyerhof, O., and Wilson, J. R., *Arch. Biochem.*, **14**, 71 (1947).
4. LePage, G. A., *J. Biol. Chem.*, **176**, 1009 (1948).
5. Schneider, W. C., *J. Biol. Chem.*, **176**, 259 (1948).
6. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis, 103 (1945).
7. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).
8. Schneider, W. C., *Cancer Res.*, **6**, 685 (1946).

AN INVESTIGATION OF THE BIOLOGICAL BEHAVIOR OF THE SULFUR ANALOGUE OF CHOLINE*

By GEORGE A. MAW† and VINCENT du VIGNEAUD

(*From the Department of Biochemistry, Cornell University Medical College, New York City*)

(Received for publication, July 16, 1948)

During the course of investigations on transmethylation involving compounds related to choline (1, 2), it was found that betaine (3) and also its sulfur analogue, dimethylthetin (4), supported growth of the white rat on a choline-methionine-free diet containing homocystine, signifying that these compounds were able to supply essential methyl groups to the body. Dimethylthetin (sulfobetaine) was of particular interest because of its being a methylsulfonium compound. In view of its remarkable growth-promoting properties when administered in a methyl-free diet to rats, the studies have been extended to include the corresponding sulfur analogue of choline, β-hydroxyethyldimethylsulfonium chloride, which we have termed for convenience "sulfocholine chloride."

EXPERIMENTAL

Preparation of Sulfocholine Chloride—Renshaw, Bacon, and Roblyer (5) described the preparation of β-hydroxyethyldimethylsulfonium iodide by the interaction of ethylene iodohydrin and dimethyl sulfide maintained at room temperature for several days. These workers noted that this method of preparation can result in the formation of considerable amounts of the trimethylsulfonium salt, due to the dissociation of the β-hydroxyethyldimethylsulfonium iodide, formed in solution, to methyl iodide and the subsequent reaction between the methyl iodide and the dimethyl sulfide present. Other non-crystallizable, oily products were also formed in this reaction. We have repeated this method of synthesis and have obtained similar results. The unavoidable formation of trimethylsulfonium salts during the preparation of methylsulfonium compounds, when dimethyl sulfide and an alkyl halide are used as starting materials, has been reported by other workers (6). We therefore describe an improved synthesis of sulfocholine iodide which obviates any formation of trimethylsulfonium iodide as a contaminant. Ethylene bromohydrin is converted to methyl β-hydroxyethyl

---

* The authors wish to express their appreciation to the Commonwealth Fund and to the Lederle Laboratories Division, American Cyanamid Company, for research grants which have aided greatly in this work.

† Commonwealth Fund Fellow; present address, Department of Biochemistry, St. Thomas's Hospital Medical School, London, England.

sulfide and the latter is treated with methyl iodide to give sulfocholine iodide in quantitative yield.

Ethylene bromohydrin (62 gm.) was added slowly to methyl mercaptan (25 gm.) dissolved in sodium methoxide (12 gm. of sodium in 200 cc. of absolute methanol). The formation of methyl β-hydroxyethyl sulfide was immediate and was completed by refluxing the product for 30 minutes. The supernatant liquid was fractionated under reduced pressure. The thio ether distilled as a colorless oil, b.p. 74–75° at 22 mm., and was halogen-free. Methyl β-hydroxyethyl sulfide (9.2 gm.) was added to methyl iodide (14.2 gm.) in a tightly stoppered bottle. The formation of the sulfonium iodide commenced within 10 minutes with the evolution of much heat and the whole reaction mixture had set to an orange crystalline mass within a further 20 minutes. The iodide was converted to the chloride without preliminary purification by shaking in aqueous solution with fresh silver chloride. On removal of the silver iodide formed, the aqueous solution was evaporated to dryness under reduced pressure and dried at 40° by means of an oil pump. The product was obtained as a slightly yellow viscous oil which crystallized to a highly deliquescent solid on being left overnight in the refrigerator.

$C_4H_{11}SOCl$.	Calculated.	C 33.7, H 7.8, S 22.5, Cl 24.9
	Found.	" 33.8, " 7.8, " 22.3, " 24.7

Sulfocholine chloroplatinate crystallized as orange needles from aqueous ethanol, capillary m.p. 190° (corrected).

Feeding Experiments—Young male albino rats of the Rockland strain were used. The animals were placed for 1 week on a preliminary diet of the following percentage composition: casein 20, fat (Covo) 19, Mazola corn oil 1, salt mixture (7) 4, sucrose 55.6, fat-soluble and water-soluble vitamins (4), L-cystine 0.4. The animals were then transferred to the experimental diet of the following composition: amino acid mixture (1)[1] 18.5, fat (Covo) 19, Mazola corn oil 1, salt mixture (7) 4, fat-soluble and water-soluble vitamins (4), DL-homocystine 0.87, L-cystine 0.4, the compound under test and sucrose to make up to 100 per cent. Sulfocholine chloride was initially fed at a level of 0.77 per cent, corresponding on the basis of methyl groups to 0.5 per cent choline chloride. In subsequent experiments lower dietary levels were also used. Total liver lipides were determined by the method of Best, Channon, and Ridout (8).

---

[1] In the amino acid mixture used, a level of 1.9 per cent L-lysine hydrochloride, together with an equivalent amount of sodium bicarbonate (0.9 per cent), was employed, and the dietary level of the mixture was 18.5 per cent.

Results

Growth Studies—Preliminary growth studies carried out on rats fed 0.77 per cent sulfocholine chloride in a methionine-choline-free diet containing homocystine showed that this compound, unlike dimethylthetin, was not able to support growth. Furthermore, it was toxic and resulted in the death of the animals in 2 to 3 weeks. Growth experiments were therefore extended to lower dietary levels and the results obtained are given in Table I and compared with those for rats on the methyl-free diet with and without added choline. At all three levels administered, sulfocholine was unable to promote growth. However, at the 0.2 per cent level there was no evidence of toxicity and over a period of 32 days the rats maintained their body weights and even gained slightly. Some maintenance of body weight was also observed in the case of other rats at the higher dietary levels over an initial period of about 1 week and before the toxic effects of the compound had become evident. This was in contrast to the behavior of the animals on the methyl-free diet. These rats suffered consistent weight losses immediately after they were placed on the experimental diet. Considering the rats on the three dietary levels as one group, it was observed that twelve of

TABLE I

Growth Rates of Rats on Methyl-Free Diets Supplemented with Sulfocholine Chloride and Methyl β-Hydroxyethyl Sulfide

Compound under test	Rat No.	Days on diet	Weight change	Food intake	Growth rate	Condition of kidneys
			gm.	gm. per day	gm. per day	
Basal methyl-free diet	50	12*	81–71	3.4	−0.8	Hemorrhagic
	25	21	113–95	5.0	−0.9	"
	28	21	97–89	4.8	−0.4	"
Sulfocholine chloride	129	32	78–89	5.2	+0.3	"
0.2%	121	32	75–85	5.3	+0.3	"
Sulfocholine chloride	122	19*	92–54	4.7	−2.0	Normal
0.39%	133	23	106–107	4.6	0.0	"
Sulfocholine chloride	44	16*	86–59	4.9	−1.7	"
0.77%	55	15*	93–65	3.9	−1.9	"
	52	21*†	105–71	5.6	−1.6	"
Methyl β-hydroxyethyl	43	12*	101–72	2.9	−2.4	Hemorrhagic
sulfide 0.99%	60	11*	89–74	3.2	−1.4	"
Choline chloride 0.5%	49	23	95–141	8.3	+2.0	Normal
	51	21	85–153	9.2	+3.2	"
	24	21	108–165	8.8	+2.7	"

* Rat died.

† Level of compound in diet reduced to 0.39 per cent after 8 days.

the nineteen animals under investigation maintained their body weight to within ±5 gm. over the first 7 days, four lost more than 5 gm., and three gained more than 5 gm. A number of the animals showed weight maintenance over longer periods than 7 days. The possible significance of this observation is discussed in a further section.

On the basis of previous studies with choline (9) and betaine (10), the inability of the sulfur analogue of choline to support the growth of rats on choline-methionine-free diets supplemented with homocystine suggests that sulfocholine cannot supply labile methyl groups for transmethylation processes. This result is somewhat surprising in view of the fact that sulfocholine may be considered as structurally intermediate between the two highly active methyl donors, choline and dimethylthetin, and may have

$$(CH_3)_3\overset{+}{N}CH_2CH_2OH \quad (CH_3)_2\overset{+}{S}CH_2CH_2OH \quad (CH_3)_2\overset{+}{S}CH_2COOH$$

been expected on this account to show some activity. It clearly emphasizes the marked structural specificity associated with the ability of a compound to act as a methyl donor (2). It is of interest that Dubnoff and Borsook (11) have reported the existence in rat and guinea pig liver of three transmethylases specifically concerned with the methylation of homocysteine by choline, betaine, and dimethylthetin, respectively.

At levels of 0.39 per cent and above in the diet, sulfocholine proved toxic to rats and resulted in their death within 2 to 3 weeks. The symptoms of toxicity were not manifest until about 1 week after the administration of the compound, although some rats during this period seemed more nervous and excitable than control animals. On about the 8th to the 10th day a black speck appeared in the corner of each eye in the neighborhood of the tear duct. This developed into an acute exudative inflammation of the anterior palpebral fissure of each eye. During the next 2 days this spread posterially along the conjunctival cutaneous junction, involving the rims of both eyelids and resulting in closure of the eyes. At this stage the corners of the mouth and occasionally the anus were found to be inflamed and encrusted and the skin of the forepaws and chest became red and inflamed. On autopsy no general gross pathology was observed, although in some rats the kidneys appeared slightly enlarged.

These toxic symptoms did not appear in rats fed 0.99 per cent methyl β-hydroxyethyl sulfide along with the methyl-free diet. However, as indicated in Table I, these animals failed to grow and died within 2 weeks, as did one of the animals on the basal diet. They were found on autopsy to have markedly hemorrhagic kidneys. This renal damage due to a methyl deficiency seems most likely to have been the cause of death. Another animal which survived on the experimental diet for 43 days likewise did not show the eye or mouth lesions described.

In view of the possibility of some of the symptoms being due to an irritant action of the compound as a result of contact with the diet, several rats were treated daily for 3 weeks with a 1 per cent aqueous solution of sulfocholine on a shaved patch of skin between the ears. No observable effect was obtained by this treatment.

The actual mechanism of the toxicity is not yet understood, but a number of possible explanations have been examined. It was considered that sulfocholine might be acting as a choline antagonist, despite the fact that the symptoms of toxicity do not resemble those of a simple choline deficiency. Rats were maintained for 6 days on the preliminary 20 per cent casein diet and were then transferred to the amino acid diet containing homocystine and supplemented by sulfocholine and choline in the following ways: 0.77 per cent sulfocholine as the chloride was fed for 10 days and 0.5 per cent choline was then added; 0.5 per cent choline was fed along with the 0.77 per cent sulfocholine for 10 days and the choline level was then raised to 1 per cent; 0.5 per cent choline was fed for 10 days before the administration of 0.77 per cent sulfocholine, and after another 10 days the choline level was raised to 1 per cent. All rats, whether given choline some days after, simultaneously with, or even prior to the addition of sulfocholine to the diet, died within 14 to 16 days.

Dimethylthetin chloride was also unable to prevent the eye and mouth lesions resulting from the feeding of sulfocholine. Rats transferred from the methyl-free diet containing 0.84 per cent dimethylthetin chloride to the same diet with added 0.6 per cent sulfocholine developed these symptoms after a period of about 10 days. Rats maintained on the preliminary 20 per cent casein diet supplemented with 0.77 per cent sulfocholine also developed the toxic symptoms, lost weight, and died within 2 weeks.

Since the inflammation of the corners of the mouth and eyes caused by sulfocholine is suggestive of a riboflavin deficiency, two rats just showing the toxic symptoms on the methyl-free diet containing 0.77 per cent sulfocholine were given 200 γ of riboflavin per day orally for 9 days. No alleviation of the condition of the eyes and mouth was observed. This would suggest that sulfocholine administration was not producing a riboflavin deficiency.

Lipotropic and Kidney Antihemorrhagic Properties—It was observed, as pointed out earlier, that, when first placed on methyl-free diets containing sulfocholine, rats did not generally suffer marked weight losses for the 1st week and in some cases for a longer period. This finding was interpreted as indicating that sulfocholine might be replacing choline in at least some of its metabolic rôles, so making available a small amount of tissue choline for transmethylation. This idea was strengthened by the finding, indicated in Tables I and II, that the kidneys of rats fed 0.39 per cent and 0.77 per cent sulfocholine were definitely protected against hemorrhagic damage. Subsequent experiments have shown that not one of the seventeen animals fed sulfocholine at these two levels developed hemorrhagic kidneys. Of seven animals fed 0.2 per cent sulfocholine, four developed hemorrhagic kidneys and three were protected. Determinations of total liver lipides were carried out on rats fed the compound at the three dietary levels, 0.2 per cent, 0.39 per cent, and 0.77 per cent, for a 10 day period. The results are shown in Table II and compared with those for rats on comparable food intakes of a methyl-free diet. Two rats given the methyl-free diet containing added choline for the same period gave total liver lipide values of 3.0 and 3.7 respectively. The results clearly demonstrate that sulfocholine is actively lipotropic at dietary levels of 0.39 per cent and above. At a level of 0.2 per cent, the lipotropic activity was only partially evident.

Table II

Total Lipides of Livers of Rats Fed Sulfocholine at Various Levels for 10 Day Period

Level of sulfocholine	Rat No.	Food intake	Weight change	Liver lipides	Condition of kidneys
per cent		*gm.*	*gm.*	*per cent wet weight liver*	
0 (Methyl-free diet)	265	48	−7	12.7	Hemorrhagic
	271	49	−12	12.9	"
	277	43	−12	9.8	"
0.2	340	60	+18	6.8	Normal
	336	45	−5	8.3	"
	328	50	−3	18.9	Hemorrhagic
0.39	276	59	0	4.8	Normal
	280	54	+3	5.2	"
	279	50	+2	3.0	"
0.77	263	46	−4	3.6	"
	278	46	−7	5.1	"
	274	45	−7	5.4	"

It might be pointed out that the protection against fatty livers and hemorrhagic kidneys was not the result of a low food intake, since the protected animals ate an amount equivalent to that consumed by the control animals.

It has been established that the lipotropic properties of arsenocholine (12, 13) and triethylcholine (14) are due to the ability of these compounds partly to replace choline in the liver phospholipides. It seemed likely that sulfocholine might be lipotropic for the same reason and an attempt was therefore made to detect sulfocholine in the liver fat of animals fed this compound. The livers were first homogenized and extracted with hot ethanol. This process would be expected to remove any unbound sulfocholine present, as well as the total lipides. The residue obtained upon evaporation of the ethanol was then extracted with hot absolute ether, thus separating the lipides from any insoluble free sulfocholine. Upon removal of the solvent, the individual liver fats were pooled in groups of three and heated in sealed tubes with excess sodium ethoxide at 70° for 3 days. Under these conditions we have found sulfocholine to be decomposed to dimethyl sulfide, as in the case of other alkyl dimethylsulfonium salts (15). The contents of the tubes were then examined for dimethyl sulfide by breaking each tube in a stout bottle connected to a chain of bubbler tubes containing saturated mercuric chloride solution. Air was drawn through the apparatus, whereupon a voluminous white precipitate was formed in the first mercuric chloride trap. This mercuric chloride complex was recrystallized from an acetone-benzene mixture. The melting point of the compound (151° (corrected)) agreed with that of the corresponding mercuric chloride complex prepared from pure dimethyl sulfide, as did the melting point of a mixture of the two substances. Analyses for sulfur and chlorine were made on the mercuric chloride derivative of the sulfide obtained from the liver fats and were compared with those for authentic dimethyl sulfide. The mercuric chloride derivative of dimethyl sulfide possessed a sulfur content of 6.45 per cent and a chlorine content of 23.2 per cent. The mercuric chloride derivative of the sulfide from the liver fats possessed a sulfur content of 6.26 per cent and a chlorine content of 23.0 per cent. The volatile sulfide thus appears to be dimethyl sulfide. Its isolation from liver fats so treated with sodium ethoxide affords evidence that sulfocholine was present in combined form in the phospholipides. This is in accord with the deductions from the analytical data in Table II and establishes the lipotropic nature of sulfocholine.

The authors wish to thank Miss Josephine E. Tietzman for performing the microanalyses and Mrs. Audrey Kellogg Hafford for technical assistance in connection with this problem.

SUMMARY

An improved synthesis of β-hydroxyethyldimethylsulfonium iodide and its conversion to the corresponding chloride are described. This latter compound, referred to as "sulfocholine chloride," has been found to be incapable of supporting the growth of rats on diets free of choline and methionine and containing homocystine. The compound was toxic above a level of 0.2 per cent in the diet. Sulfocholine has been found to be active in preventing the development of fatty livers and renal hemorrhages in rats fed the methyl-free diet.

A volatile sulfide has been isolated from the livers of such rats by a

procedure which is known to cause the degradation of sulfocholine to dimethyl sulfide. The sulfide has been identified as dimethyl sulfide. This is presented as evidence that the lipotropic activity of sulfocholine is due to its incorporation into liver phospholipides in place of choline.

BIBLIOGRAPHY

1. du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939).
2. du Vigneaud, V., *Harvey Lectures*, **38**, 39 (1942–43).
3. Chandler, J. P., and du Vigneaud, V., *J. Biol. Chem.*, **135**, 223 (1940).
4. du Vigneaud, V., Moyer, A. W., and Chandler, J. P., *J. Biol. Chem.*, **174**, 477 (1948).
5. Renshaw, R. R., Bacon, N., and Roblyer, J. H., *J. Am. Chem. Soc.*, **48**, 517 (1926).
6. Ingold, C. K., Jessop, J. A., Kuriyan, K. I., and Mandour, A. M. M., *J. Chem. Soc.*, 533 (1933).
7. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).
8. Best, C. H., Channon, H. J., and Ridout, J. H., *J. Physiol.*, **81**, 409 (1934).
9. Simmonds, S., Cohn, M., Chandler, J. P., and du Vigneaud, V., *J. Biol. Chem.*, **149**, 519 (1943).
10. du Vigneaud, V., Simmonds, S., Chandler, J. P., and Cohn, M., *J. Biol. Chem.*, **165**, 639 (1946).
11. Dubnoff, J. W., and Borsook, H., *Federation Proc.*, **7**, 152 (1948).
12. Welch, A. D., *Proc. Soc. Exp. Biol. and Med.*, **35**, 107 (1936).
13. Welch, A. D., and Landau, R. L., *J. Biol. Chem.*, **144**, 581 (1942).
14. McArthur, C. S., *Science*, **104**, 222 (1946).
15. Gleave, J. L., Hughes, E. D., and Ingold, C. K., *J. Chem. Soc.*, 236 (1935).

COMPOUNDS RELATED TO DIMETHYLTHETIN AS SOURCES OF LABILE METHYL GROUPS*

By GEORGE A. MAW† AND VINCENT DU VIGNEAUD

(*From the Department of Biochemistry, Cornell University Medical College, New York City*)

(Received for publication, August 3, 1948)

Previous studies carried out in this laboratory (1–4) have shown that the only compounds so far known to be capable of supporting the growth of the rat on a choline-methionine-free diet supplemented with homocystine are choline itself (and choline derivatives, *e.g.* lecithin and phosphorylcholine), dimethylethyl-β-hydroxyethylammonium chloride (monoethylcholine), betaine, and dimethylthetin (sulfobetaine). On the basis of experiments involving the feeding of deuteriocholine and deuteriobetaine to rats (5, 6), the activity of compounds in supporting growth under these conditions is interpreted as signifying that they are able to transfer methyl groups to homocystine to form methionine. In addition to promoting growth, these methyl-donating compounds are also able to prevent the formation of fatty livers (3). However, other compounds devoid of available methyl groups are active lipotropic agents, and thus the ability to act as a lipotropic agent cannot be considered synonymous with the ability to act as a donor of essential methyl groups (7).

In view of the pronounced methyl-donating activity of the sulfur analogue of betaine (4) and the lack of this activity in the case of the corresponding sulfur analogue of choline (8), it became of interest to extend these investigations to other sulfonium compounds in order to gain further information of the structural specificity involved. Compounds tested included methylethylthetin, diethylthetin, and dimethyl-β-propiothetin. The last named compound appeared of special significance, since it has recently been isolated from a marine alga, *Polysiphonia fastigiata*, by Challenger and Simpson (9). In a preliminary communication we have already reported it to be an excellent substitute for choline in methyl-free diets containing homocystine (10).

EXPERIMENTAL

Preparation of Compounds—S-Methylthioglycolic acid was prepared by methylation of ethyl thioglycolate with methyl iodide in alcoholic sodium

---

* The authors wish to express their appreciation to the Commonwealth Fund and to the Lederle Laboratories Division, American Cyanamid Company, for research grants which have aided greatly in this work.

† Commonwealth Fund Fellow; present address, Department of Biochemistry, St. Thomas's Hospital Medical School, London, England.

methoxide and hydrolysis of the resulting ester with sodium hydroxide. Dimethylthetin, methylethylthetin, and diethylthetin were obtained as their chlorides by the interaction of monochloroacetic acid with dimethyl, methylethyl, and diethyl sulfides, respectively, in nitromethane as solvent. Dimethyl-β-propiothetin chloride was prepared by treatment of β-iodopropionic acid with dimethyl sulfide in nitromethane and conversion of the resulting sulfonium iodide to the chloride by shaking with dry silver chloride in absolute ethanol.

Feeding Experiments—Young male albino rats of the Rockland strain were used. The animals were placed on a preliminary diet containing 20 per cent casein (Diet I, composition given in the following section). After being kept on this diet for approximately 1 week they were transferred for 7 days to a diet containing pure amino acids as the protein component (1)[1] together with 1 per cent DL-methionine (Diet II). The rats showing most satisfactory growth at this stage (a growth rate of 2 to 3 gm. per day) were then selected for the feeding of the compounds under investigation. The animals were transferred to a diet similar to Diet II, with the exception that the methionine was replaced by 0.87 per cent DL-homocystine together with the compound under test (Diet III).

Composition of Diets—The percentage composition of the diets was as follows: Diet I, casein 20, fat (Covo) 19, sucrose 55.6, salt mixture (11) 4, Mazola corn oil 1, fat-soluble and water-soluble vitamins as reported in a previous publication (4), L-cystine 0.4. Diet II, amino acid mixture (1)[1] 18.5, sucrose 56.1, DL-methionine 1.0, L-cystine 0.4, fat, salt mixture, and vitamins as in Diet I. Diet III, amino acid mixture (1)[1] 18.5, DL-homocystine 0.87, L-cystine 0.4, fat, salt mixture, and vitamins as in Diet I, the compound under test and sucrose to make up to 100 per cent. Methylthioglycolic acid, dimethylthetin chloride, and dimethylpropiothetin chloride were administered in amounts corresponding on the basis of methyl groups to an arbitrarily chosen level of 0.5 per cent choline chloride. Methylethylthetin chloride and diethylthetin chloride were given in amounts equivalent on a molar basis to the dimethylthetin chloride fed.

Preparation of S-Trideuteriomethylthioglycolic Acid—Ethyl thioglycolate (2.4 gm.) was dissolved in sodium methoxide (0.46 gm. of sodium in 7 cc. of absolute methanol) at −10°, and 3.0 gm. of trideuteriomethyl iodide (12) were added gradually. The resulting solution gave a negative test for the sulfhydryl grouping. The methanol was evaporated, the residue was heated with 3 cc. of water, and the ethyl deuteriomethylthioglycolate was saponified by the dropwise addition of sufficient 15 per cent potassium hydroxide to keep the reaction mixture alkaline to brom-thymol blue. The solution was then acidified with concentrated hydrochloric acid and the trideuteriomethylthioglycolic acid was extracted with two 7 cc. portions of ether, dried over sodium sulfate, and distilled at 112–113° at a pressure of 12 mm.

---

[1] In the amino acid mixture used, a level of 1.9 per cent of L-lysine hydrochloride, together with an equivalent amount of sodium bicarbonate (0.9 per cent), was employed, and the dietary level of the mixture was 18.5 per cent.

Analyses—Trideuteriomethylthioglycolic acid

Elementary.[2] Calculated, S 29.77; found, S 29.71

Deuterium. 53.1 ± 0.7 atom % excess in methyl group

Feeding Experiment—Two 85 gm. rats were maintained on the preliminary diet (Diet I) for 6 days and then were transferred for 11 days to Diet III together with 1.14 per cent S-trideuteriomethylthioglycolic acid and 0.9 per cent sodium bicarbonate to neutralize the acid. (The addition of the base was effective in reducing almost completely the odor of the compound in the diet.) Food intakes were determined daily and further supplements of the sodium salt of trideuteriomethylthioglycolic acid in water were given by stomach tube in order to make up the daily intake to 70 mg. or to that amount which would be supplied in 6 gm. of diet. Since the food intakes were 4 to 5 gm. per day, the amount administered by stomach tube represented only a small fraction of the total dose. At the end of the period the rats were sacrificed. Carcass choline was isolated as the chloroplatinate, creatine as creatinine potassium picrate (12), and both compounds were analyzed for deuterium (13).

Results

In Table I are shown the growth rates and food intakes of rats fed the various compounds under discussion in methyl-free diets containing homocystine. For comparison the corresponding data for rats on a methyl-free diet with and without the choline supplement are given. Dimethylthetin and dimethylpropiothetin were clearly able to support growth as well as choline itself and produced no apparent toxic effects at the dietary levels used. The rats maintained on these compounds remained in excellent health throughout the experiment and in no case was there a detectable growth lag during the transfer from the methionine-containing diet to the diet containing homocystine and the compound under test. Methylethylthetin appeared to be somewhat less active as a methyl donor. Two of the rats on this compound grew as well as those on dimethylthetin; four animals grew at a slower rate although they fared substantially better than those on the methyl-free diet; one died after 10 days. Diethylthetin was inactive in supporting growth.

[2] The calculated value is based on the increased molecular weight due to deuterium in the molecule.

Both dimethylthetin and dimethylpropiothetin prevented the formation of hemorrhagic kidneys in the animals studied. Methylethylthetin definitely prevented hemorrhagic kidneys in five out of the seven animals.

Table I

Growth Rates and Food Intakes of Rats Fed Dimethylthetin and Related Compounds

Compound under test	Rat No.	Days on diet	Weight change	Food intake	Growth rate	Condition of kidney
			gm.	*gm. per day*	*gm. per day*	
Basal methyl-free diet	3650	27	96–69	4.1	−1.0	Hemorrhagic
	3654	27	99–89	4.6	−0.4	"
	3660	10*	103–86	4.3	−1.7	"
	50	12*	81–71	3.4	−0.8	"
	25	21	113–95	5.0	−0.9	"
	28	21	97–89	4.8	−0.4	"
Choline 0.5%	3643	23	93–139	8.6	+2.0	Normal
	49	23	95–141	8.3	+2.0	"
	51	21	85–153	9.2	+3.2	"
	24	21	108–165	8.8	+2.7	"
Dimethylthetin 0.84%	3644	24	90–150	7.4	+2.5	"
	3658	24	107–186	9.2	+3.3	"
	3661	27	78–124	6.6	+1.7	"
Dimethylpropiothetin 0.92%	41	21	98–158	8.8	+2.9	"
	48	21	88–137	7.9	+2.3	"
	57	21	101–180	9.3	+3.8	"
Methylethylthetin 0.92%	3649	16	88–94	5.7	+0.4	"
	3651	10*†	103–71	4.0	−3.2	"
	3653	16	83–104	6.9	+1.3	"
	3656	23	89–145	9.4	+2.4	"
	47	21	84–93	5.1	+0.4	?
	54	23	96–136	6.7	+1.7	Hemorrhagic
	26	21	109–163	8.1	+2.6	Normal
Diethylthetin 0.99%	3645	27	85–61	3.5	−0.9	Hemorrhagic
	3655	27	93–79	5.2	−0.5	"
	3657	27	105–84	4.9	−0.8	"
S-Methylthioglycolic acid 0.5%	3646	13*	89–75	3.5	−1.1	"
	3659	27	104–93	5.2	−0.4	"
S-Methylthioglycolic acid 1.14%	3648	14*	103–58	3.4	−3.2	"
	3652	27	85–63	3.5	−0.8	"

* Rat died.

† Cause of death unknown.

In another rat of this group, protection was doubtful. Diethylthetin was completely ineffective.

Dimethylthetin has previously been reported as being lipotropic by Welch, as referred to by Moyer and du Vigneaud (3). In Table II are shown parallel data obtained from rats given methylethylthetin and dimethylpropiothetin. The liver fat values are compared with the corresponding figures for rats on a methyl-free diet with and without added choline. Dimethylpropiothetin was highly active as a lipotropic agent, as would be expected from its ability to promote growth on homocystine diets and to prevent renal hemorrhages. Methylethylthetin was again not as active as dimethylthetin, the former compound protecting four out of the six rats from fatty livers.

TABLE II

Total Liver Lipide Values

Compound under test	Rat No.	Days on diet	Food intake	Total liver lipides
			gm. per day	*per cent wet weight liver*
Basal methyl-free diet	25	21	5.0	16.6
	28	21	4.8	20.6
Choline 0.5%	3643	23	8.6	4.7
	24	21	8.8	4.8
	51	21	9.2	4.1
Dimethylpropiothetin 0.92%	57	21	9.3	5.2
	41	21	8.8	5.2
	48	21	7.9	4.0
Methylethylthetin 0.92%	3649	16	5.7	4.4
	3653	16	6.9	5.3
	3656	23	9.4	5.5
	47	21	5.1	12.5
	54	23	6.7	5.6
	26	21	8.1	10.9

TABLE III

*Feeding Experiment with S-Trideuteriomethylthioglycolic Acid**

Rat No.	Change in body weight	Choline† isolated		Creatine isolated	
		Deuterium in methyl groups	Per cent derived from compound fed	Deuterium in methyl groups	Per cent derived from compound fed
39	102–92	0.49 ± 0.25	0.91	0.52 ± 0.40	0.99
21	99–88	0.52 ± 0.13	0.99	0.37 ± 0.39	0.70

* The experiments were conducted over an 11 day period.

† Analyses for choline chloroplatinate: Rat 39, calculated, Pt 31.6, found, 31.8; Rat 21, calculated, Pt 31.6, found, 32.2 per cent.

As shown in Table I, S-methylthioglycolic acid, the sulfur counterpart of dimethylglycine, was unable to support growth at the two levels fed. The higher level corresponds on the basis of methyl groups to 0.5 per cent choline, but since it was considered that the compound might be toxic at such a concentration a lower level was also used. The compound was also unable to prevent the onset of hemorrhagic kidneys.

In view of the close structural relationship of S-methylthioglycolic acid to dimethylthetin, it seemed possible that methylthioglycolic acid might be capable of acting as a precursor of dimethylthetin in the body, thus acting as an indirect methyl donor. It may be recalled that dimethylaminoethanol (14) has been shown to act as an indirect methyl donor, being unable to promote growth appreciably, yet able to furnish methyl groups which eventually find their way into tissue creatine and choline.

To investigate the rôle of methylthioglycolic acid, this compound was prepared with its methyl group labeled with deuterium and was fed to two rats. After a period sufficiently long for detectable transmethylation to have taken place, the rats were killed and the tissue choline and creatine were analyzed for deuterium. The results are shown in Table III. Less than 1 per cent of the deuteriomethyl groups was found to be present in the choline and creatine and in the case of the latter compound the amount present was within the range of experimental error. The extent of this methyl transfer is comparable to that obtained from sarcosine (15) and dimethylglycine (6), both regarded as poor methyl donors. These results indicate that methylthioglycolic acid is not an active source of methyl groups. In addition, the results suggest that the methylation of methylthioglycolic acid to dimethylthetin does not take place to an appreciable extent in the body under the conditions described.

DISCUSSION

The demonstration by du Vigneaud, Moyer, and Chandler of the ability of dimethylthetin to promote the growth of rats on methyl-free diets (4), together with the present findings of the similar activity of dimethylpropiothetin (10), clearly marks these methylsulfonium compounds as a new class of methyl donors. These *in vivo* experiments are in agreement with the independent *in vitro* experiments of Dubnoff and Borsook (16), who have shown dimethylthetin[3] to be an extremely active methyl donor for homocysteine in liver homogenates. The earlier observation of Welch (3) that dimethylthetin is a lipotropic agent and our data that methylethylthetin and dimethylpropiothetin are also lipotropic add further support. In addition, Welch[4] has reported that dimethylthetin and methylethylthetin are able to protect animals on methyl-free diets from renal hemorrhagic damage. Our results are in confirmation and also include dimethylpropiothetin as an antihemorrhagic agent.

---

[3] In the oral presentation of their paper on dimethylthetin (meeting of the American Society of Biological Chemists at Atlantic City, March 15, 1948), the abstract of which is referred to, Dubnoff and Borsook reported that dimethylpropiothetin was also active in promoting methionine synthesis.

[4] Welch, A. D., private communication.

The position of methylethylthetin as a methyl donor appears to be a border line one and intermediate between dimethylthetin and diethylthetin, since it was capable of supporting good growth in only two out of the seven animals under test. The compound was able to protect all but two of the animals from fatty livers and all but two from renal hemorrhagic damage. Its lower activity compared with dimethylthetin may possibly be associated with the presence in the molecule of an ethyl group bound in a similar manner to the methyl group. It has previously been observed that the replacement of the methyl groups in choline and methionine by ethyl groups leads to decreased growth-promoting activity and increased toxicity (3, 17).

Diethylthetin was unable to support growth and animals fed on this compound developed hemorrhagic kidneys. Welch has found it to be inactive as a lipotropic agent.[4] Triethylcholine, also devoid of methyl groups, is known, however, to be a lipotropic and antihemorrhagic agent on account of its ability to replace the choline molecule as a whole in certain of its metabolic rôles (3, 18). Diethylthetin must therefore be unable to replace the entire choline molecule or to form a further compound in the body which is effective. In this respect it resembles arsenobetaine and phosphobetaine (19).

The original discovery by Challenger and Simpson of the presence of dimethylpropiothetin in *Polysiphonia fastigiata* (9), which prompted growth studies on this compound, introduces the possibility that substances of this type may be present in other organisms. Moreover, the fact that dimethylthetin and its homologue have been found to be active methyl donors in the rat coupled with the report of Dubnoff and Borsook (16) that an enzyme specifically catalyzing the methylation of homocysteine by dimethylthetin exists in rat and guinea pig liver tissue would suggest that either dimethylthetin or a homologue or closely related derivative may actually be a tissue constituent and may take part in normal methylation processes. If this is the case, such compounds would have to be considered as potential dietary factors, and a diet deficient in methionine, choline, and betaine alone could no longer be considered as methyl-free unless methyl-donating thetins had been shown to be absent.

Many of the earlier studies on the metabolism of choline, betaine, and related compounds have been directed towards modification of the structure of the compound in question in order to determine to what extent the molecular configuration may be altered without effecting a loss in biological activity. With lipotropism as the biological activity in mind, it has been possible, in the case of choline, to make considerable structural changes, including complete replacement of methyl by ethyl groups (18), and replacement of the nitrogen atom by phosphorus (19), arsenic (20), and sulfur (8) with retention of the lipotropic properties. The most reasonable explanation at the present time seems to be that the various substituted cholines are being utilized in the liver for phospholipide formation in lieu of choline itself and are active merely by virtue of being choline analogues.

In the case of methyl-donating ability, if the assumption is made that dimethylthetin is active because it is the sulfur analogue of betaine, then one might expect sulfocholine to be at least as effective, if not more so. However, it has been shown not to be a methyl donor and to be toxic above a certain dietary level (8). Furthermore, whereas dimethylpropiothetin exhibits a marked growth-promoting activity, its nitrogen analogue, β-alanine betaine, is toxic and apparently not a methyl donor (3). The concept of the methylsulfonium compounds under discussion as being methyl donors simply on account of their structural similarity to nitrogen compounds already known to take part in transmethylation reactions would seem misleading, particularly in view of the existence of a separate enzyme system concerned with transmethylation from these compounds (16). The thetins referred to must be considered as methyl donors in their own right and not because of their analogy to betaine.

From an examination of the structures of the six compounds which have been shown to be capable of methylating homocystine *in vivo*, namely choline, monoethylcholine, betaine, dimethylthetin, methylethylthetin, and dimethylpropiothetin, it appears that the structural criterion associated with this process is the presence, in the molecule of the potential methyl donor, of a methyl group or groups directly attached to an onium pole. Growth-promoting activity is not exhibited by compounds lacking either a methyl group or an onium structure, although lipotropic properties may still be exhibited by such compounds. On the one hand, triethylcholine (1, 3) and diethylthetin, lacking available methyl groups, are inactive in promoting growth. On the other hand, N-methyl and S-methyl compounds such as methylaminoethanol and dimethylaminoethanol (14), sarcosine (15), dimethylglycine (6), methyl β-hydroxyethyl sulfide (8), and S-methylthioglycolic acid, which lack the onium structure, are likewise inactive in promoting growth. This criterion for methylation activity is further coupled with an enzymic specificity, since many other methylated onium compounds, including arsenocholine (21), N-methylnicotinamide (22), trigonelline (3), and various betaines (3) are ineffective in this respect.

SUMMARY

Growth studies on rats fed methionine-choline-free diets supplemented with homocystine together with a number of compounds related to di-

methylthetin show that, in addition to the latter compound, dimethylpropiothetin is a highly active methyl donor. Methylethylthetin is less active and diethylthetin is quite inactive.

Dimethylpropiothetin is an effective lipotropic and kidney antihemorrhagic agent, methylethylthetin is again less active, and diethylthetin shows no protective properties.

S-Methylthioglycolic acid is unable to support growth or to protect animals against renal hemorrhagic damage. Its inability to act as an efficient methyl donor to homocystine has been confirmed by labeling the S-methyl group with deuterium. Inappreciable amounts of the isotope were found to be present in the methyl groups of tissue choline and creatine after 11 days.

The relationship of methyl-donating ability to chemical structure is discussed.

BIBLIOGRAPHY

1. du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131,** 57 (1939).
2. Chandler, J. P., and du Vigneaud, V., *J. Biol. Chem.*, **135,** 223 (1940).
3. Moyer, A. W., and du Vigneaud, V., *J. Biol. Chem.*, **143,** 373 (1942).
4. du Vigneaud, V., Moyer, A. W., and Chandler, J. P., *J. Biol. Chem.*, **174,** 477 (1948).
5. Simmonds, S., Cohn, M., Chandler, J. P., and du Vigneaud, V., *J. Biol. Chem.*, **149,** 519 (1943).
6. du Vigneaud, V., Simmonds, S., Chandler, J. P., and Cohn, M., *J. Biol. Chem.*, **165,** 639 (1946).
7. du Vigneaud, V., *Harvey Lectures*, **38,** 39 (1942–43).
8. Maw, G. A., and du Vigneaud, V., *J. Biol. Chem.*, **176,** 1029 (1948).
9. Challenger, F., and Simpson, M. I., *Biochem. J.*, **41,** p. xl (1947).
10. Maw, G. A., and du Vigneaud, V., *J. Biol. Chem.*, **174,** 381 (1948).
11. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37,** 572 (1919).
12. du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S., *J. Biol. Chem.*, **140,** 625 (1941).
13. Keston, A. S., Rittenberg, D., and Schoenheimer, R., *J. Biol. Chem.*, **122,** 227 (1937–38).
14. du Vigneaud, V., Chandler, J. P., Simmonds, S., Moyer, A. W., and Cohn, M., *J. Biol. Chem.*, **164,** 603 (1946).
15. du Vigneaud, V., Simmonds, S., and Cohn, M., *J. Biol. Chem.*, **166,** 47 (1946).
16. Dubnoff, J. W., and Borsook, H., *Federation Proc.*, **7,** 152 (1948).
17. Dyer, H. M., *J. Biol. Chem.*, **124,** 519 (1938).
18. Channon, H. J., and Smith, J. A. B., *Biochem. J.*, **30,** 115 (1936).
19. Welch, A. D., and Welch, M. S., *Proc. Soc. Exp. Biol. and Med.*, **39,** 7 (1938).
20. Welch, A. D., *Proc. Soc. Exp. Biol. and Med.*, **35,** 107 (1936).
21. Welch, A. D., *J. Biol. Chem.*, **137,** 173 (1941).
22. Keller, E. B., Wood, J. L., and du Vigneaud, V., *Proc. Soc. Exp. Biol. and Med.*, **67,** 182 (1948).

BIOSYNTHESIS OF PENICILLINS*

VIII. STUDIES WITH NEW BIOSYNTHETIC PENICILLINS ON PENICILLIN RESISTANCE

By OTTO K. BEHRENS and MARY JANE KINGKADE

(*From the Lilly Research Laboratories, Indianapolis*)

(Received for publication, July 24, 1948)

It has been reported that the natural penicillins show a similar specificity of action on various microorganisms. Eisman (1) has demonstrated that a *Staphylococcus* which had acquired resistance to benzylpenicillin (penicillin G) was also resistant to *p*-hydroxybenzyl- and 2-pentenylpenicillins (penicillins X and F). However, several investigators have observed that the relative activity of the penicillins was not regular, but varied from organism to organism (*e.g.* (2)). It was of interest to determine whether the activity of the new biosynthetic penicillins (3) would fall in a similar narrow range of variation, or whether such penicillins, containing acyl groups derived from biologically foreign substances, would exhibit a wider range of action.

Resistance of organisms to penicillin has been ascribed to at least two different mechanisms. One of these involves production of penicillinase either as an intracellular or extracellular enzyme. The other does not appear to involve penicillinase. In determining the specificity of action of the new penicillins, tests were, therefore, conducted (*a*) by comparing the rates of reaction of these compounds with penicillinase, and (*b*) by determining their effectiveness against a benzylpenicillin-resistant strain of *Staphylococcus aureus* 209-P which did not produce demonstrable quantities of penicillinase.

EXPERIMENTAL

Reactions of Penicillinase with Some Biosynthetic Penicillins—The manometric method of Henry and Housewright (4) for assaying penicillin proved to be a simple and relatively precise procedure for following the penicillin-penicillinase reactions. The following solutions were used in the determinations.

1. Sodium bicarbonate buffer, pH 7.0, prepared by dissolving 357.1 mg. of sodium bicarbonate (Baker) in 500 ml. of distilled water and equilibrating the solution with a gas mixture of 95 per cent O_2-5 per cent CO_2.

---

* For paper VII, see Soper, Q. F., Whitehead, C. W., Behrens, O. K., Corse, J., and Jones, R. G., *J. Am. Chem. Soc.*, **70**, 2849 (1948).

2. Penicillinase solution, prepared immediately before use by dissolving 0.7 mg. of penicillinase[1] in 12.5 ml. of the bicarbonate buffer.

3. Penicillin solutions, prepared by dissolving the sodium salt of the penicillin in enough bicarbonate buffer so that 0.5 ml. of solution contained 1×10^{-5} mole of the sodium penicillin. The concentrations of penicillin solutions were checked by bioassay.

Constant volume Warburg respirometers were used. The main compartment of the vessel contained 2.5 ml. of bicarbonate buffer and 0.5 ml. of penicillin solution. The system was equilibrated with the gas mixture at 37.5°, closed, and the enzyme solution (0.5 ml.) from the side arm was tipped into the reaction vessel. The rate of carbon dioxide evolution was followed for 100 minutes. Duplicate or triplicate determinations were made on each penicillin in comparison with benzylpenicillin.

Table I

Destruction of Penicillins by Penicillinase

Penicillin, sodium salt	No. of assays	*k* ratio*
Phenoxymethyl-	2	1.40
β-Phenoxyethylmercaptomethyl-	15	1.17
β-Bromoallylmercaptomethyl-	4	1.17
α-Thiophenemethyl-	4	1.15
m-Trifluoromethylphenylmercaptomethyl-	2	1.14
Allylmercaptomethyl-	6	1.08
o-Fluorobenzyl-	2	1.07
Ethylmercaptomethyl-	6	1.05
p-Bromobenzyl-	2	1.02
Isopropylmercaptomethyl-	6	1.02
Phenylselenomethyl-	2	1.01
Benzyl-	37	1.00
p-Methoxybenzyl-	1	0.98
Isoamylmercaptomethyl-	9	0.97
n-Butylmercaptomethyl-	9	0.92
n-Propylmercaptomethyl-	6	0.92
m-Fluorobenzyl-	2	0.90
p-Tolylmethyl-	2	0.83
Cyclopentylmethyl-	4	0.79

* (Rate of reaction of penicillinase and new penicillin) (rate of reaction of penicillinase with benzylpenicillin). The values represent the average of the several determinations.

The rates of CO_2 evolution varied considerably from one run to another, presumably reflecting differences in enzyme concentration. However, the ratio of the rates of reaction of an experimental penicillin to that of benzylpenicillin (*k* ratio) was reproducible to ±6 per cent in all but one case. The *k* ratio varied from 0.79 for cyclopentylmethylpenicillin to 1.40 for phenoxymethylpenicillin (*cf.* Table I). Thus, though the biosynthetic penicillins reacted with penicillinase at significantly different rates, the differences were not great enough to be of therapeutic importance.

---

[1] Penicillinase A, furnished by the courtesy of Dr. George E. Ward of the Schenley Laboratories, Inc., Lawrenceburg, Indiana.

Effect of Some Biosynthetic Penicillins on Benzylpenicillin-Resistant Staphylococcus aureus—A strain resistant to 200 units per ml. of benzylpenicillin in broth culture was developed by serial transfers of *Staphylococcus aureus* 209-P through broth and on agar plates containing increasing concentrations of penicillin.

TABLE II

Effectiveness of Biosynthetic Penicillins on Benzylpenicillin-Resistant Strain of Staphylococcus aureus

Penicillin (Na salt)	Purity	Average relative effectiveness
	per cent	
α-Thiophenemethyl-	95	1.7
p-Bromobenzyl-	89	1.6
m-Fluorobenzyl-	100	1.2
p-Methoxybenzyl-	99	1.0
Ethylmercaptomethyl-	98	1.0
Benzyl-	100	1.0
o-Fluorobenzyl-	94	0.96
Cyclopentylmethyl-	96	0.62
p-Tolylmethyl-	96	0.62
Phenylselenomethyl-	98	0.60
n-Propylmercaptomethyl-	98	0.54
Isopropylmercaptomethyl-	97	0.47
β-Bromoallyl-	91	0.41
Allylmercaptomethyl-	96	0.40
Phenoxymethyl-	90	0.35
n-Butylmercaptomethyl-	98	0.32
Isoamylmercaptomethyl-	94	0.26
β-Phenoxyethylmercaptomethyl-	98	0.21

Penicillin assays were performed by the plate method (5) with paper disks (6) by use of the resistant strain of *Staphylococcus aureus* as inoculum. An 0.018 M solution of benzylpenicillin (10,000 units per ml.) produced an 18.8 mm. zone.

Concentrations of penicillin were plotted against diameters of the corresponding zones of inhibition. The ratios of moles of benzylpenicillin and biosynthetic penicillins giving equal zones were determined at three or four points on the curve and the average ratios were calculated (*cf.* Table II).

Generally, the relative activities of the new penicillins decreased as concentrations increased. However, ethylmercaptomethylpenicillin and *p*-methoxybenzylpenicillin were less effective than benzylpenicillin at lower concentrations but became more effective at 0.045 M (20,000 units per ml.) and 0.026 M (15,000 units per ml.) concentration respectively.

The resistant organism apparently did not produce penicillinase. Filtrates from nutrient broth cultures did not inactivate benzylpenicillin in 4 hours at room temperature. 10 mg. per cent suspensions of cells which had been extracted with acetone and ether did not enable a sensitive strain of *Staphylococcus aureus* to survive in increased concentrations of penicillin.

Table II also gives figures concerning the purity of the penicillins tested. These values represent the ratio of the found analytical value to the calculated analytical value for the analysis which deviated most from the theoretical value. In the penicillins which contained a unique group that could be determined (*i.e.*, OCH_3, Br, Se), the ratio should be an accurate measure of purity. In other penicillins, for which C, N, or S determinations were utilized, this measure of purity is only approximate, as some possible contaminating materials also may contain these elements.

In spite of the fact that the resistance was induced by subculturing in the presence of benzylpenicillin, this penicillin was one of the most effective in preventing growth of the organism.

No correlations could be made between the action of the new penicillins with penicillinase and the bacteriostatic action of the penicillins on benzylpenicillin-resistant *Staphylococcus aureus*.

The authors express their gratitude to Dr. J. M. McGuire for assistance with media and assays and to E. Brown Robbins for assistance with the manometric work.

SUMMARY

1. The rate of destruction of a number of biosynthetic penicillins by penicillinase has been compared with the rate of destruction of benzylpenicillin.

2. The relative effectiveness of a number of biosynthetic penicillins on a strain of benzylpenicillin-resistant *Staphylococcus aureus* has been determined.

BIBLIOGRAPHY

1. Eisman, P. C., Marsch, W. S., and Mayer, R. L., *Science*, **103**, 673 (1946).
2. Welch, H., Putman, L. E., Randall, W. A., and Herwick, R. P., *J. Am. Med. Assn.*, **126**, 1024 (1924).
3. Behrens, O. K., Corse, J., Edwards, J. P., Garrison, L., Jones, R. G., Soper, Q. F., Van Abeele, F. R., and Whitehead, C. W., *J. Biol. Chem.*, **175**, 793 (1948).
4. Henry, R. J., and Housewright, R. D., *J. Biol. Chem.*, **167**, 559 (1947).
5. Schmidt, W. H., and Moyer, A. J., *J. Bact.*, **47**, 199 (1944).
6. Loo, Y. H., Skell, P. S., Thornberry, H. H., Ehrlich, J., McGuire, J. M., Savage, G. M., and Sylvester, J. C., *J. Bact.*, **50**, 701 (1945).

THE RÔLE OF TRYPTOPHAN IN THE NUTRITION OF DOGS ON NICOTINIC ACID-DEFICIENT DIETS*

BY S. A. SINGAL, V. P. SYDENSTRICKER, AND JULIA M. LITTLEJOHN

(From the Departments of Biochemistry and Medicine, University of Georgia School of Medicine, Augusta)

(Received for publication, July 23, 1948)

It has now been definitely established that there exists an interchangeability of nicotinic acid and tryptophan in the nutrition of some animals on diets deficient in the vitamin and containing only suboptimal amounts of the amino acid (1–8).

Because the dog has been employed as the experimental animal in the classical investigations on nicotinic acid deficiency, it seemed of interest to determine whether this same nicotinic acid-tryptophan relationship exists for this species. The results of these nutrition experiments, together with those obtained in the study of the urinary excretion of some nicotinic acid derivatives under these experimental conditions, are presented here.

EXPERIMENTAL

Twenty-two weanling, mongrel puppies, 7 to 8 weeks old, were used in these studies. The basal ration consisted of casein (Labco) 19, sucrose 66, salts (9) 4, and cottonseed oil 11 parts. All rations were fed *ad libitum* and each dog received in addition, per kilo of body weight per day, 100 γ each of thiamine, riboflavin, and pyridoxine, 500 γ of calcium pantothenate, and 50 mg. of choline chloride. Folic acid and biotin were administered at a level of 30 and 20 γ, respectively, per dog per day. The required amounts of vitamins were given in 20 per cent ethyl alcohol solutions twice weekly. Vitamins A and D were given once weekly in the form of halibut liver oil fortified with viosterol at a level of 3 drops per kilo of body weight. Supplements of casein, zein, and gelatin to the basal diet replaced an equal amount of sucrose.

The 24 hour urine specimens were collected under toluene. The hydrolysis of urine was accomplished by autoclaving at 15 pounds for 15 minutes an aliquot of urine with an equal volume of either 2.0 N H_2SO_4 or NaOH. The nicotinic acid content of the specimens and hydrolysates was determined microbiologically by the method of Snell and Wright (10). N^1-

---

* Presented in part at the meetings of the Federation of American Societies for Experimental Biology, Chicago, 1947 (*Federation Proc.*, **6**, 422 (1947)). Acknowledgment is made of aid from the John and Mary R. Markle Foundation and the United States Public Health Service. Folic acid was generously contributed by the Lederle Laboratories Division, American Cyanamid Company.

Methylnicotinamide was determined by the method of Huff and Perlzweig (11).

Nutrition Studies

Growth data of dogs on nicotinic acid-deficient diets are presented in Table I. It is evident that upon the institution of the basal diet dogs gain an average of 540 gm. before reaching a growth plateau in about 12 days. Thereafter drastic weight loss is usually rapid and other symptoms of blacktongue, characterized by inflammation of the gums, palatine redness, and diarrhea, usually develop if therapy is withheld. To reduce the loss of dogs, nicotinic acid or tryptophan was usually administered after the dogs refused food for 48 hours. Frequently, severe symptoms had al-

TABLE I

Growth of Dogs on Nicotinic Acid-Deficient Diets

Diet	Dog No.	Weight gain	Days to reach plateau	Weight gain after nicotinic acid*	
		gm.		*gm.*	*gm. per mg. nicotinic acid*
Basal	1 ♂	540	8	960	38
	3 ♀	740	15	1240	38
	9 ♀	520	11	800	26
	10 ♀	600	14	960	33
	11 ♂	520	14	920	27
	16 ♂	460	14	1380	37
	20 ♂	420	8	1000	30
Average.........		540	12	1050	33
Basal + 21% zein	6 ♀	620	15	1080	36
	17 ♂	780	18	1200	39
Average.........		700	17	1140	38
Basal + 21% gelatin	5 ♀	560	11	700	22
	18 ♂	630	16	750	25
Average.........		600	14	725	24
Basal + 21% casein	7 ♀	1100	21	1400	46
	19 ♀	1500	24	1650	50
	21 ♂	1450	37		
Average.........		1350	27	1525	48

* A single dose of nicotinic acid at a level of 10.0 mg. per kilo was administered.

ready developed. When one therapeutic dose of nicotinic acid was given at a level of 10.0 mg. per kilo of body weight, the dogs resumed intake of food within 24 hours and gained on an average 1140 gm. before reaching another weight plateau. This amounts to 33 gm. gain in weight per mg. of nicotinic acid given.

The development of nicotinic acid deficiency in dogs on the basal diet is not prevented or significantly altered by the inclusion of the tryptophan-deficient proteins, zein or gelatin, in the diet. Two dogs receiving the basal ration supplemented with 21 per cent zein gained an average of 700 gm. before reaching a growth plateau in 17 days. With a therapeutic dose of nicotinic acid 1140 gm. were added to the body weight, or an average of 38 gm. per mg. of nicotinic acid administered. The two dogs on the basal diet supplemented with 21 per cent gelatin show similar increases in body weight before growth plateau. With nicotinic acid, however, the growth response is somewhat smaller, as is evident from the smaller value of gm. gained per mg. of nicotinic acid given.

The addition of 21 per cent casein to the basal diet does not prevent the onset of nicotinic acid deficiency, but significantly delayed its appearance as judged by the much larger weight gain before the weight plateau was reached and the longer period of time necessary to reach the plateau. In two of these animals this plateau persisted for 17 and 21 days before drastic decline in weight occurred. The plateau of animals on the basal diet lasted from 3 to 12 days. The response to nicotinic acid is also larger than that observed on the other diets. In the third animal, after a gain of 1450 gm., the growth plateau persisted for 30 days. Nicotinic acid was given when there was no indication of drastic weight loss. At first the animal gained weight steadily and during the next 60 days growth was erratic, but there was no significant tendency for the animal to reach a weight plateau.

With supplementary L-tryptophan at a level of 0.5 per cent, one dog (Fig. 1, Dog 2) received complete protection against the onset of nicotinic acid deficiency for an experimental period of 60 days. This animal grew at a rate comparable to that of animals receiving nicotinic acid at a level of 500 γ per kilo of body weight per day in addition to the basal diet. Inasmuch as a casein supplement of 21 per cent to the basal diet did not afford protection, the supplement was increased to 42 per cent, at which level the tryptophan content of the entire diet was equivalent to that of the basal diet supplemented with 0.5 per cent L-tryptophan.[1] This high level of protein in the diet afforded complete protection to two dogs for periods of 74 and 100 days, the length of the experimental periods (Fig. 1, Dog 12). In these periods the dogs gained 8.1 and 9.0 kilos in body weight.

---

[1] The tryptophan content of casein was taken as 1.2 per cent (12).

Inasmuch as further experiments with L-tryptophan were inadvisable because of the cost of the natural amino acid, DL-tryptophan was employed in the remaining experiments of the study. As with the L form, the racemic amino acid at a level of 0.5 per cent prevents the occurrence of nicotinic acid deficiency in the dog (Fig. 1, Dog 8). Levels of 0.3, 0.2, and 0.1 per cent were then fed to determine the minimal protective level. It is apparent that this minimum must be less than 0.1 per cent, since dogs at this level are fully protected (Fig. 1, Dog 13). After a gain in body weight of 6.3 kilos distemper appeared in one dog and the experiment was terminated. It is, however, apparent that 0.1 per cent DL-tryptophan affords protective

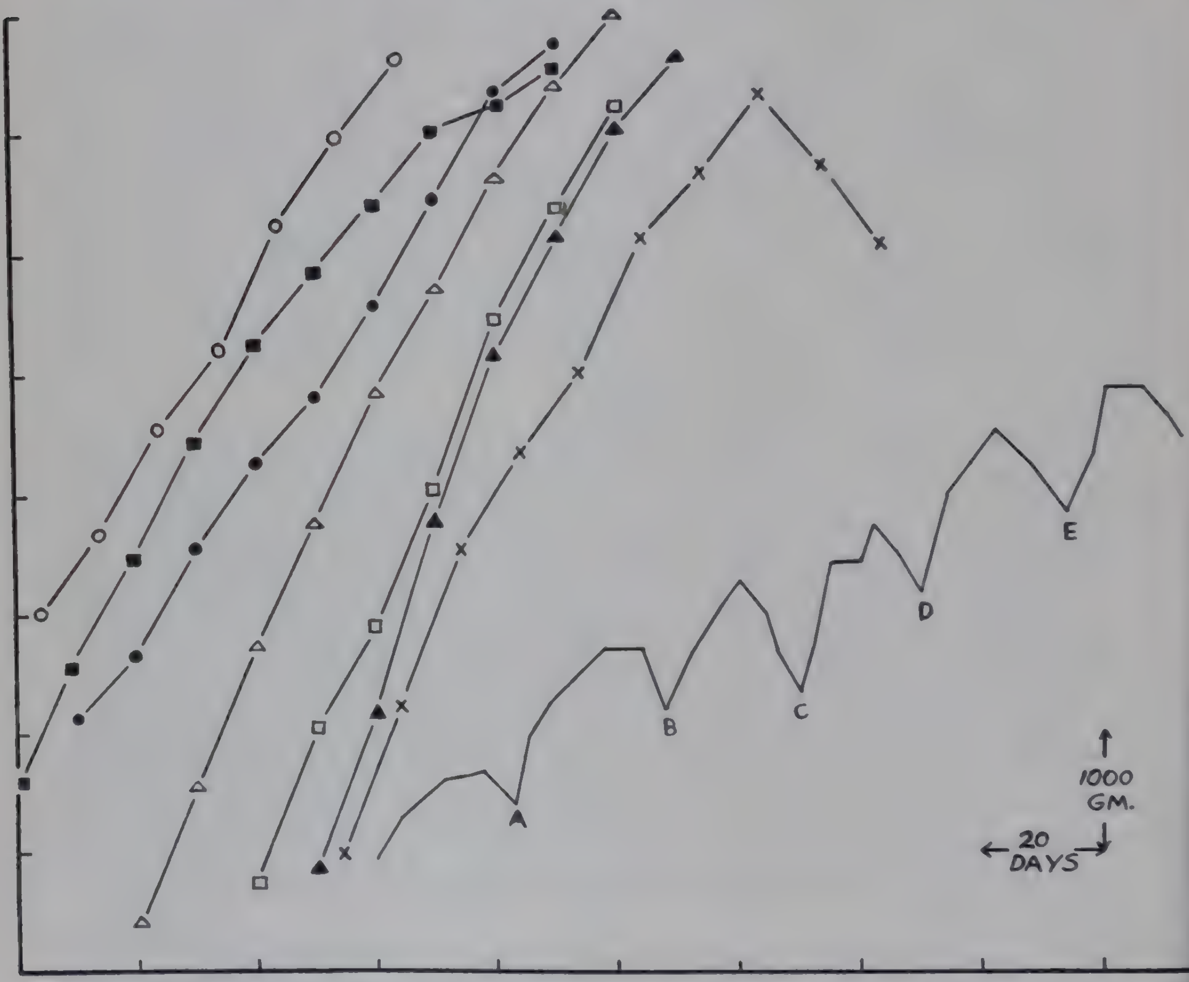


Fig. 1. The growth of dogs on various diets. ○, Dog 2 ♀, basal diet + 0.5 per cent L-tryptophan; ●, Dog 8 ♂, basal + 0.5 per cent DL-tryptophan; ▲, Dog 15 ♀, basal + 0.3 per cent DL-tryptophan; □, Dog 14 ♀, basal + 0.2 per cent DL-tryptophan; ×, Dog 13 ♂, basal + 0.1 per cent DL-tryptophan; △, Dog 12 ♀, basal + 42 per cent casein; ■ Dog 4 ♂, basal + 500 γ of nicotinic acid per kilo of body weight daily. The lower right-hand curve represents Dog 3 ♀, basal diet; 33 mg. of nicotinic acid given at *A*, *C*, and *E*; 3.96 and 5.66 gm. of L-tryptophan at *B* and *D*, respectively.

action. It is of interest to note that, although the basal diet supplemented with 21 per cent casein containing a total of 0.48 per cent tryptophan[1] is ineffective in preventing nicotinic acid deficiency, the basal diet supplemented with 0.1 per cent DL-tryptophan containing a total of 0.28 per cent tryptophan[2] is effective in this respect. One must assume, therefore, under these experimental conditions that supplementary tryptophan must be metabolized in a manner different from tryptophan present as an integral part of the protein molecule. It also appears unlikely that tryptophan present in the protein molecule with other amino acids is more readily utilized for the synthesis of tissue protein, a process which would remove it as a source of nicotinic acid synthesis. The protein requirement of the dog is met by a level of 19 per cent casein, because supplementary nicotinic acid promotes normal growth under these conditions (Fig. 1, Dog 4). This leaves unaccounted for the metabolic route of the protein tryptophan in the 21 per cent casein supplement. One might consider that the free amino acid is more rapidly absorbed than protein tryptophan because of the delay necessitated by the hydrolytic process in the gastrointestinal tract and thereby metabolized in the absence of large amounts of other amino acids present in the protein. In this respect it would be of interest to compare the protective action of supplementary casein with that of an enzyme hydrolysate of casein.

At the present time there is not sufficient evidence to account for the protective action of high levels of supplementary casein (42 per cent). It is certainly not related to the nicotinic acid content of the protein *per se*. By microbiological assay a casein hydrolysate prepared with trypsin contains only 0.17 γ of nicotinic acid per gm. of casein. The two dogs, each consuming an average of 300 gm. of diet daily, would obtain only 31 γ of nicotinic acid in this manner. With an initial body weight of 3.0 kilos, the intake would be only 10 γ per kilo of body weight. This is less than one-twentieth of the minimal requirement (13).

In view of the fact that L- and DL-tryptophan had protective action at the levels fed in these experiments, it was of interest to determine the curative potency of the D and L forms in terms of nicotinic acid. This was performed by comparing the growth-promoting effect of a single dose of the L- and DL-amino acid in nicotinic acid-deficient dogs, which had been standardized with known amounts of nicotinic acid (13). The procedure was altered in that the assay of the amino acid was preceded and followed by a standardization response to nicotinic acid. An example of this assay is shown in Fig. 1. The results of these experiments are presented in Table

---

[2] The calculation includes the tryptophan content of the casein of the basal diet and in addition one-half of the supplementary DL-tryptophan, as only the L form is utilized for nicotinic acid synthesis (see Table II).

II. It is apparent that only the natural form is utilized for nicotinic acid synthesis. The curative effect of L-tryptophan failed in three assays. In one the administration of nicotinic acid promptly stopped the drastic weight loss and a growth response was then obtained which was in excess of that expected from nicotinic acid alone. In the other two animals amounts of nicotinic acid as high as 500 mg. failed to check the rapid decline. In one of these nasal hemorrhage was observed. The administration of 500 mg. of ascorbic acid and 2.0 mg. of 2-methyl-1,4-naphthoquinone intramuscularly was without effect and the two animals died in an extremely emaciated condition 9 days later.

TABLE II

*Nicotinic Acid Equivalence of L- and DL-Tryptophan**

Values in mg. per gm. of amino acid.

Dog. No.	L-Tryptophan	DL-Tryptophan
3	5.6	Plateau†
	6.1	3.0
5	6.1	Plateau
7	9.0	"
9	10.0	"
	8.1	5.1
11	7.7	
16	8.2	3.7

* Tryptophan was administered at a level of 1200 mg. per kilo of body weight in two portions during a 24 to 48 hour period. To prevent vomiting of the supplement, the amino acid was ground with an equal weight of sucrose and given either in gelatin capsules or as a paste.

† The term plateau indicates that the drastic weight loss ceased and that the body weight was maintained for at least 7 days.

Nicotinic Acid Excretion Studies

In the rat the administration of tryptophan is followed by the urinary excretion of relatively large amounts of nicotinic acid derivatives (14–18). In Table III are given the results obtained in the dog. Change of diet from commercial dog food to the synthetic diet is reflected in a rapid fall in the excretion of the nicotinic acid derivatives measured. This is less marked in the nicotinic acid values obtained after acid hydrolysis. In fact, by the 2nd day complete recovery has been obtained. Thereafter the values rise steadily to rather large levels. By contrast nicotinic acid values obtained in untreated or alkaline-hydrolyzed urine after the initial drop regain only a third of their original values. Since nicotinic acid and its amide have equal activities for the assay organism, *Lactobacillus arabi-*

nosus, and nicotinuric acid does not require preliminary hydrolysis to show the same activity as its theoretical equivalent of nicotinic acid (10), it appears that after the administration of tryptophan to dogs, as in rats (15), there is excreted a nicotinic acid precursor which is converted to nicotinic acid by acid hydrolysis. This substance makes up the major portion of the total nicotinic acid derivatives excreted. By comparison N^1-methylnicotinamide, which is the major excretory substance in animals on a commercial animal ration, is only temporarily excreted in large amounts in dogs on synthetic diets containing tryptophan (days 3 to 8). Following this period the values are not in excess of those found in dogs receiving nicotinic acid (Table IV). In the rat tryptophan stimulates the excretion of relatively large amounts of nicotinic acid, nicotinic acid precursor, and N^1-methylnicotinamide, whereas in the dog only the excretion of the acid-hydrolyzable nicotinic acid precursor is marked. The pattern of excretion of nicotinic acid derivatives is markedly altered in dogs when the commercial ration is replaced by the basal diet supplemented with nicotinic acid (Table IV). In the former the excretion of nicotinic acid is the same regardless of the treatment of the urine, indicating that the substances excreted resemble the simple derivatives, nicotinic acid, nicotinamide, or nicotinuric acid. N^1-Methylnicotinamide accounts for 87 per cent of the total nicotinic acid derivatives excreted. By comparison, on the synthetic

TABLE III

Urinary Excretion of Nicotinic Acid Derivatives in Dog Receiving Basal Diet Supplemented with 0.5 Per Cent L-*Tryptophan*

Days on diet	Nicotinic acid			N^1-Methylnicotinamide
	No hydrolysis	Acid hydrolysis	Alkaline hydrolysis	
	γ	γ	γ	γ
0*	655	720	692	2650
1	100	414	137	1072
2	84	700	64	206
3	208	1240	208	1460
5	205	1120	200	1410
8	302	1560	305	2710
16	153	1848	113	861
25	120	1830	103	600
31	225	2340	182	709
40	191	2170	191	615
46	192	2240	222	579
56	240	2660	178	573

* Excretion values on this day represent those of the animal on a commercial animal ration. Thereafter, the experimental diet was instituted.

diet the values of nicotinic acid excretion are dependent on the treatment of urine before assay. Similar values are obtained in untreated or alkaline-hydrolyzed urine. With acid treatment the apparent nicotinic acid is increased by as much as a 3-fold factor. This difference may indicate that the dog on the synthetic ration excretes small amounts of the acid-hydrolyzable precursor encountered in animals receiving tryptophan.[3] The excretion of N^1-methylnicotinamide is also markedly reduced in the change of ration. The 6-pyridone of N^1-methylnicotinamide (19, 20) may also play an important rôle in excretion of nicotinic acid derivatives in the dog. It has recently been reported that this substance is the major pathway of excretion of dietary nicotinic acid in the human (21). Similarly, it may account for the major fraction of methylated derivatives of nicotinic acid in dogs receiving tryptophan or nicotinic acid.

TABLE IV

Urinary Excretion of Nicotinic Acid Derivatives in Dog Receiving Basal Diet and 0.5 Mg. of Nicotinic Acid per Kilo of Body Weight per Day

Days on diet	Nicotinic acid			N^1-Methylnicotinamide
	No hydrolysis	Acid hydrolysis	Alkaline hydrolysis	
	γ	γ	γ	γ
0*	588	573	513	3880
1	394	470	338	1530
2	88	224	75	234
3	100	220	80	233
5	114	275	88	350
8	114	326	93	341
16	142	324	121	412
25	118	330	81	442
31	201	375	213	505
40	155	344	121	569
50	193	400	152	502
61	210	470	185	613

* Excretion values on this day represent those of the animal on a commercial animal ration. Thereafter, the experimental diet was instituted.

The excretion of nicotinic acid derivatives in a dog on a high casein diet is shown in Table V. The protective action of this diet is apparently related to the synthesis of nicotinic acid as judged by the increased excre-

---

[3] The nature of the nicotinic acid fraction in the rat is similarly affected by the replacement of commercial animal ration with a synthetic diet. On the former, rats excrete nicotinic acid, the value of which is the same regardless whether the urine has been hydrolyzed with acid or alkali or left untreated before assay. Upon change to the synthetic diet, the urinary nicotinic acid is always somewhat greater in acid-hydrolyzed specimens.

TABLE V

Urinary Excretion of Nicotinic Acid Derivatives in Dog Receiving Basal Diet Supplemented with 42 Per Cent Casein

Days on diet	Nicotinic acid			$\mathrm{N^L}$-Methylnicotinamide
	No hydrolysis	Acid hydrolysis	Alkaline hydrolysis	
	γ	γ	γ	γ
0*	598	630	610	2700
1	102	585	107	1100
2	161	962	177	649
3	181	846	180	1422
5	210	1039	207	1666
8	167	880	189	1166
25	255	763	261	1607
27	244	807	240	2431

* Excretion values on this day represent those of the animal on a commercial animal ration. Thereafter, the experimental diet was instituted.

TABLE VI

Effect of L*-Tryptophan and Nicotinic Acid on Urinary Excretion of Nicotinic Acid Derivatives in Dog with Blacktongue (Dog 9)*

Days on diet	Nicotinic acid			$\mathrm{N^L}$-Methylnicotinamide
	No hydrolysis	Acid hydrolysis	Alkaline hydrolysis	
	γ	γ	γ	γ
0*	140	366	108	52
1†	84	472	66	186
2†	172	890	156	1150
3	134	950	107	1080
4	136	452	112	469
5	110	356	81	482
6	95	305	70	96
7	107	250	90	104
8	112	278	88	91
0*	108	228	91	107
1‡	4210	4220	4400	3630
2	119	209	112	656
3	140	270	127	566
4	98	161	84	140
5	153	290	131	391

* Excretion values on these days represent those of the animal in a nicotinic acid-deficient state.

† 2.0 gm. of L-tryptophan were given in a capsule.

‡ 35 mg. of nicotinic acid were given in a capsule.

tion of the acid-labile precursor and N^1-methylnicotinamide. The appearance of the latter in amounts greater than that found either in dogs on the basal diet supplemented with nicotinic acid (Table IV) or in the over-all excretion period in dogs receiving tryptophan will require further study for explanation.

In the nicotinic acid-deficient dog relatively large amounts of tryptophan can stimulate the increased excretion of the acid-hydrolyzable precursor of nicotinic acid and N^1-methylnicotinamide (Table VI). In six studies four animals have shown these increased values. The failure of the other two has been interpreted as retention of the nicotinic acid synthesized from tryptophan. It would be well also to consider the efficiency of this synthesis in the deficient animal. When receiving therapeutic amounts of nicotinic acid, the deficient dog excretes only 23 per cent of the administered dose as nicotinic acid and N^1-methylnicotinamide. The nicotinic acid precursor does not appear to be excreted in significant amounts after the administration of nicotinic acid as judged by the similar values of nicotinic acid excretion obtained in untreated and acid- and alkaline-hydrolyzed urine. This may imply that the precursor represents an intermediate in the synthesis of nicotinic acid and not a mechanism to remove a surplus of the vitamin in the body. Its excretion in dogs receiving tryptophan would indicate that its conversion to nicotinic acid is at a lower rate thar the methylation of the nicotinic acid to N^1-methylnicotinamide or mor likely the 6-pyridone derivative.

DISCUSSION

The interchangeable rôle of nicotinic acid and tryptophan has alread been demonstrated in the nutrition of the rat (1, 2, 5–7), mouse (4), chic (3), and pig (8, 22). Our results indicate that this relationship also exist for the dog. Urinary excretion studies lend support to the rôle of tryptophan as the biological precursor of nicotinic acid (7, 14–18).

The level of casein in the diet necessary to abolish the requirement fo nicotinic acid varies from 20 to 25 per cent for the rat (7, 23) and the pi (8, 22) to a value in excess of 40 per cent for the dog as reported here. I these latter results are applicable to human nutrition, some question obviously raised as to the relative importance of protein and dietary nic tinic acid in meeting the nicotinic acid requirement. It would appe rather unlikely that this level of protein is within the average dietary experience (24). This would emphasize the importance of nicotinic acid diets of moderate protein content in which the protein is derived to a lar extent from animal sources (24). These foods contain large amounts the vitamin (25).

Rather large quantities of milk have been successful in the treatment pellagra (26). In the rat its beneficial action has been ascribed to its protein content, since the amount of nicotinic acid present is very low (2). In our own studies 5 ml. of milk per day were ineffective in preventing growth retardation in rats on low protein diets supplemented with gelatin.[4] Experiments with the nicotinic acid-deficient dog have indicated that milk has a greater beneficial action than can be ascribed to its nicotinic acid content as determined microbiologically (13). In our own hands this procedure has given rather unreliable results.

The presence of a substance in milk similar to nicotinic acid in its biological activity should be considered in view of the fact that milk, the major food consumed after birth, contains little nicotinic acid. On a dry basis human milk contains only 10 per cent protein (27), which is insufficient for nicotinic acid synthesis in the rat and pig, and certainly a quantity considerably less than that required by the dog. Salmon (28) has shown that the nicotinic acid requirement of the rat is abolished on low protein diets containing 30 per cent fat. In view of the fact that on the dry basis milk contains 28 per cent fat, we may have here a possible explanation for the absence of nicotinic acid deficiency in the nursing young.

SUMMARY

The interchangeable rôle of nicotinic acid and tryptophan in the nutrition of dogs has been studied. At a level of 21 per cent in a nicotinic acid-deficient ration, gelatin, zein, or casein does not prevent the onset of nicotinic acid deficiency in the dog. However, when the casein supplement was increased to 42 per cent, complete protection was obtained.

Supplementary L- or DL-tryptophan at the 0.5 per cent level similarly prevents the occurrence of the deficiency syndrome. The minimal protective level of DL-tryptophan is less than 0.1 per cent, as dogs on the deficient ration supplemented with 0.3, 0.2, or 0.1 per cent of the amino acid grow at the normal rate and are without deficiency symptoms during the experimental period.

The nicotinic acid equivalence of L- and DL-tryptophan has been determined in nicotinic acid-deficient animals, which have been standardized with known amounts of nicotinic acid. By this method it has been found that only the natural isomer is utilized for nicotinic acid synthesis.

The urinary excretion of some nicotinic acid derivatives in the dog has been studied. The administration of L-tryptophan results in the excretion of an acid-hydrolyzable precursor of nicotinic acid, which has previously been reported present in the urine of rats under similar conditions. There is only a temporary increase in urinary N^1-methylnicotinamide. Dogs on rations supplemented with 42 per cent casein also excrete larger amounts of the precursor and N^1-methylnicotinamide than did dogs growing normally on the basal ration supplemented with nicotinic acid. The results of the excretion and nutrition studies are interpreted as indicating that the synthesis of nicotinic acid from tryptophan does take place in this species.

---

[4] Unpublished results.

BIBLIOGRAPHY

1. Krehl, W. A., Teply, L. J., and Elvehjem, C. A., *Science*, **101**, 283 (1945).
2. Krehl, W. A., Teply, L. J., and Elvehjem, C. A., *Science*, **101**, 489 (1945).
3. Briggs, G. M., *J. Biol. Chem.*, **161**, 749 (1945).
4. Woolley, D. W., *J. Biol. Chem.*, **162**, 179 (1946).
5. Spector, H., and Mitchell, H. H., *J. Biol. Chem.*, **165**, 37 (1946).
6. Krehl, W. A., Sarma, P. S., and Elvehjem, C. A., *J. Biol. Chem.*, **162**, 403 (1946).
7. Singal, S. A., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, **171**, 203 (1947).
8. Luecke, R. W., McMillen, W. N., Thorp, F., Jr., and Tull, C., *J. Nutr.*, **33**, 251 (1947).
9. Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109**, 657 (1935).
10. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
11. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **167**, 157 (1947).
12. Horn, M. J., and Jones, D. B., *J. Biol. Chem.*, **157**, 153 (1945).
13. Schaefer, A. E., McKibbin, J. M., and Elvehjem, C. A., *J. Biol. Chem.*, **144**, 679 (1942).
14. Rosen, F., Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **163**, 343 (1946).
15. Singal, S. A., Briggs, A. P., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, **166**, 573 (1946).
16. Hundley, J. M., *J. Nutr.*, **34**, 253 (1947).
17. Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, **168**, 555 (1947).
18. Spector, H., *J. Biol. Chem.*, **173**, 659 (1948).
19. Knox, W. E., and Grossman, W. I., *J. Biol. Chem.*, **166**, 391 (1946).
20. Knox, W. E., and Grossman, W. I., *J. Biol. Chem.*, **168**, 363 (1947).
21. Rosen, F., Perlzweig, W. A., and Handler, P., *Federation Proc.*, **7**, 181 (1948).
22. Wintrobe, M. M., Stein, H. J., Follis, R. H., Jr., and Humphreys, S., *J. Nutr.*, **30**, 395 (1945).
23. Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, **172**, 485 (1948).
24. Booher, L. E., in Sahyun, M., Proteins and amino acids in nutrition, New York (1948).
25. Snell, E. E., and Wright, L. D., *Univ. Texas Pub., No. 4137*, 22 (1941).
26. Goldberger, J., and Tanner, W. F., *Pub. Health Rep., U. S. P. H. S.*, **39**, 87 (1924).
27. Holt, L. E., Jr., and McIntosh, R., Holt's diseases of infancy and childhood, New York, 11th edition (1939).
28. Salmon, W. D., *J. Nutr.*, **33**, 155 (1947).

FURTHER STUDIES ON THE EFFECT OF SOME AMINO ACIDS ON THE GROWTH AND NICOTINIC ACID STORAGE OF RATS ON LOW CASEIN DIETS*

By S. A. SINGAL, V. P. SYDENSTRICKER, and JULIA M. LITTLEJOHN

(*From the Departments of Biochemistry and Medicine, University of Georgia School of Medicine, Augusta*)

(Received for publication, July 23, 1948)

In a previous communication from this laboratory it was shown that the inclusion of an amino acid mixture containing histidine, valine, threonine, and lysine in a low protein diet produced marked depression of growth in rats (1). The addition of either nicotinic acid or tryptophan not only corrected the growth retardation, but permitted normal growth, which was not possible in the absence of these amino acids. That lysine was not the amino acid responsible for the growth depression was evident from the fact that its omission from the amino acid mixture did not significantly alter the results obtained.

In the present paper the investigation has been continued to determine which amino acids are growth depressants and which are required by the rat for normal growth on low casein diets supplemented with nicotinic acid or tryptophan. Data are presented which indicate that threonine is the amino acid effective in both categories.

EXPERIMENTAL

Wistar strain rats, 22 days old, were used in these experiments. The basal diet consisted of casein (Labco) 9, sucrose 82, salts (2) 4, L-cystine 0.2, cottonseed oil 3, and cod liver oil 2 parts. Vitamins were incorporated in 100 gm. of diet at the following levels: thiamine 1.0 mg., riboflavin 1.0 mg., pyridoxine 1.0 mg., calcium pantothenate 2.0 mg., choline chloride 200 mg., 2-methyl-1,4-naphthoquinone 0.5 mg., inositol 10 mg., biotin 0.02 mg., and folic acid 0.2 mg. α-Tocopherol was administered at a level of 1.0 mg. per rat per week. Supplements of amino acids replaced an equal amount of sucrose in the diet. At first they were incorporated in the diet as pairs and later their effects were studied individually.

The nicotinic acid content of muscle and liver was determined microbiologically (3) on tissue extracts prepared by autolysis aided by takadiastase and papain (4).

---

* Acknowledgment is made of aid from the John and Mary R. Markle Foundation and the United States Public Health Service. Folic acid and biotin were generously contributed by the Lederle Laboratories Division, American Cyanamid Company, and Merck and Company, Inc., respectively.

From the results in Table I it is evident that growth on the basal ration can be improved to a limited, yet reproducible, extent by the addition of either nicotinic acid or tryptophan. This is reflected in an increased nicotinic acid content of muscle and liver, although the amino acid is much more effective in this respect. Under these experimental conditions it is possible to maintain nicotinic acid storage that is near normal with supplementary tryptophan in spite of suboptimal growth (Diets 3 to 5).

When threonine and valine are added to the basal ration, there is observed a marked growth depression, which is not reflected in a concomitant decrease in nicotinic acid storage (Diet 6). On the contrary, values are obtained which are usually in excess of those found for rats on the basal diet alone. This may be explained on the basis of a decreased demand on the nicotinic acid stores of the rats on threonine-containing diets, because of the small amount of new tissue laid down. Supplementary nicotinic acid or tryptophan not only corrects the inhibition, but permits normal growth, which is not possible in the absence of these amino acids (Diets 7 and 8). Liver nicotinic acid is nearly normal in animals receiving dietary nicotinic acid, whereas there is no considerable change in muscle storage. The inability of dietary nicotinic acid to raise substantially muscle storage toward normal has been observed in all threonine-containing diets (Nos. 7, 13, and 16), in spite of reasonably good growth. With supplementary tryptophan muscle nicotinic acid is in the normal range. Liver storage is considerably above normal, a finding previously reported (1). Perhaps this indicates an accumulation in the liver of either nicotinic acid or intermediates in the synthesis of nicotinic acid from tryptophan, which can replace the nicotinic acid requirement of the test organism, *Lactobacillus arabinosus*, employed in the microbiological assays. Apparently not involved is the acid-hydrolyzable derivative of nicotinic acid previously reported present in the urine of rats receiving tryptophan (1, 5).

The amino acid pair containing valine and histidine, when added to the basal ration, does not significantly alter either the growth or nicotinic acid storage of rats on the basal ration alone (Diet 9). Supplementary nicotinic acid or tryptophan produces the same effects with this diet as with the basal diet alone (Diets 10 and 11).

With threonine and histidine there is obtained a growth inhibition which resembles that observed in animals on the diet containing threonine and valine (Diet 12). A supplement of nicotinic acid or tryptophan also produces similar effects in regard to growth and nicotinic acid storage (Diets 13 and 14). In consideration of the results obtained with diets containing pairs of amino acids, it is apparent that growth inhibition is observed only when threonine is one member of the pair. In addition the basal ration contains only suboptimal amounts of this amino acid, because when it is added to the ration, either nicotinic acid or tryptophan can stimulate optimal growth.

TABLE I

Growth and Muscle and Liver Nicotinic Acid of Rats on Various Diets

Diet No.	No. of rats	Diet*	Gain per wk. for 4 wks.	Nicotinic acid	
				Muscle	Liver
			gm.	*γ per gm.*	*γ per gm.*
1	7	Stock	25 (22–29)†	74 (61–85)†	159 (132–185)†
2	7	20% casein	25 (21–33)	78 (70–85)	186 (167–206)
3	11	Basal	9 (8–12)	38 (28–52)	86 (57–114)
4	11	" + 2.0 mg. % nicotinic acid	13 (10–16)	73 (64–90)	111 (63–165)
5	11	" + 0.2 % L-tryptophan	14 (9–17)	79 (65–94)	150 (70–227)
6	8	" + threonine + valine	2 (0– 3)	46 (34–64)	137 (64–217)
7	8	Diet 6 + 2.0 mg. % nicotinic acid	22 (18–27)	50 (44–59)	152 (150–166)
8	8	" 6 + 0.2 % L-tryptophan	24 (21–28)	77 (61–87)	228 (171–303)
9	8	Basal + valine + histidine	10 (8–12)	37 (30–48)	90 (43–110)
10	8	Diet 9 + 2.0 mg. % nicotinic acid	14 (11–15)	68 (56–80)	109 (67–138)
11	8	" 9 + 0.2 % L-tryptophan	13 (10–17)	80 (63–92)	144 (77–208)
12	8	Basal + threonine + histidine	2 (1– 3)	45 (28–74)	125 (77–164)
13	8	Diet 12 + 2.0 mg. % nicotinic acid	19 (15–25)	53 (47–61)	146 (111–164)
14	8	Diet 12 + 0.2 % L-tryptophan	23 (20–25)	86 (77–95)	222 (190–254)
15	9	Basal + threonine	3 (0– 6)	41 (23–67)	131 (93–200)
16	9	Diet 15 + 2.0 mg.% nicotinic acid	19 (16–24)	45 (30–58)	141 (106–186)
17	9	Diet 15 + 0.2 % L-tryptophan	22 (19–27)	70 (56–85)	229 (179–281)
18	8	Basal + valine	9 (8–12)	42 (26–63)	82 (61–109)
19	8	Diet 18 + 2.0 mg. % nicotinic acid	13 (10–15)	64 (44–81)	99 (59–122)
20	8	Diet 18 + 0.2 % L-tryptophan	9 (9–11)	78 (60–88)	127 (83–195)
21	8	Basal + histidine	9 (8–10)	40 (31–60)	84 (58–131)
22	8	Diet 21 + 2.0 mg. % nicotinic acid	12 (9–15)	62 (54–70)	96 (88–107)
23	8	Diet 21 + 0.2 % L-tryptophan	12 (8–16)	79 (65–92)	141 (88–178)
24	5	Basal + phenylalanine	9 (7–11)	41 (34–45)	84 (73– 90)
25	5	Diet 24 + 2.0 mg. % nicotinic acid	12 (8–14)	63 (50–71)	101 (71–123)
26	5	Diet 24 + 0.2 % L-tryptophan	12 (8–14)	80 (64–90)	138 (116–184)

* The stock ration consists of commercial animal food supplemented liberally with milk, lettuce, and carrots. The levels of supplementary amino acids are DL-threonine 0.4 per cent, DL-valine 0.3 per cent, L-histidine monohydrochloride 0.25 per cent, and DL-phenylalanine 0.25 per cent.

† The values in parentheses represent the range.

The effects of the individual amino acids were then studied to determine whether the presence of either valine or histidine was required for the activity of threonine. It is evident that the growth-depressing action of the amino acid mixture is entirely confined to threonine. Valine and histidine are without effect individually, as they are when included together in the basal ration (Diets 18 to 23). It is difficult to explain the inability of tryptophan to produce in the presence of valine the limited growth response observable on the basal ration plus tryptophan (compare Diets 20 and 5). With supplementary nicotinic acid or tryptophan, threonine alone is required for reasonably good growth (Diets 16 and 17). It is of interest to note that at the level fed nicotinic acid is a little less effective than tryptophan. When the requirement of tryptophan is divided between that necessary for protein synthesis and that needed for nicotinic acid synthesis, it may well be that the amount of the amino acid present in the basal ration alone is not quite sufficient for the former process when nicotinic acid and threonine are included in the ration. When nicotinic acid is replaced by tryptophan, this requirement is easily met. At smaller growth rates, in which protein synthesis is already limited by the amount of threonine present in the basal ration, these differences disappear.

DISCUSSION

Krehl *et al.* (5, 6) first observed growth retardation in rats on low protein diets containing large amounts of corn. The addition of either tryptophan or nicotinic acid corrected the deficiency and permitted normal growth to proceed. Briggs extended this observation to the chick and further demonstrated that a similar depression could be obtained by the addition of gelatin to a purified diet (7, 8). In this work the inhibitory effect was not noted when gelatin was replaced by arginine and glycine. Later work from this laboratory indicated that the two amino acids were growth-inhibitory under somewhat altered conditions (9). The addition of alanine furthered the depressant effect. The feeding of an amino acid mixture, simulating the relative occurrences of some amino acids in gelatin, produced an inhibition observed with the protein itself. The omission of arginine and glycine from the mixture permitted good growth. In more recent results Anderson and Briggs observed that the inhibitory effect of some seventeen amino acids varied from the rather deleterious effect of methionine to a 20 per cent inhibition by valine (10).

In extension of their original observations the University of Wisconsin workers have found that it was not necessary to postulate a specific pellagragenic agent in corn for the rat, at least, because the deficiency syndrome could be duplicated with non-corn rations by the addition of tryptophan-deficient proteins, such as zein or gelatin, or acid-hydrolyzed proteins to a nicotinic acid-deficient diet low in tryptophan (11). Of a number of amino acids tested for growth inhibition, glycine was particularly effective (12). Supplementary nicotinic acid removed the inhibition, but normal growth was not obtained. The deleterious effect of acid-hydrolyzed casein could be reproduced with a mixture of amino acids made to simulate the hydrolysate. That this inhbition was not due to glycine and alanine *per se* was evident from the fact that their omission from the mixture did not prevent growth inhibition (13).

Beginning with the suggestion that the usual 9 per cent casein diet is deficient in a number of amino acids (14), the present authors observed that the addition of a mixture of amino acids containing lysine, valine, threonine, and histidine to a low protein diet produced marked growth retardation in rats (1). Supplementary nicotinic acid or tryptophan not only corrected the deficiency, but also produced good growth, which was not possible in the absence of these amino acids. The omission of lysine from the mixture did not significantly alter the data. The results of the present report indicate that of the remaining members of the mixture valine and histidine do not alter growth significantly. Threonine alone is responsible for the growth inhibition by the mixture. Furthermore, it is revealed that this amino acid is the limiting factor for growth on the low casein diet. Recently, this latter observation has also been made by Griffith (15).

Salmon (16) in his studies has stated that the primary deficiency on low casein diets was that of labile methyl groups and, secondly, nicotinic acid. Only after the requirement of the vitamin was satisfied, could a deficiency of sulfur amino acid be demonstrated. Under our conditions, in which the basal low protein diet is already supplemented with choline and cystine, the growth-promoting action of nicotinic acid is limited only by the suboptimal amounts of threonine in the basal ration.

Niven *et al.* (17) have observed marked growth retardation in rats receiving phenylalanine and tyrosine. The addition of relatively large amounts of nicotinic acid or tryptophan corrected only in part the deficiency syndrome. Our results indicate that at a 0.25 per cent level phenylalanine does not have inhibitory action, nor can it promote good growth with supplementary nicotinic acid or tryptophan (Diets 24 and 25).

The nature of the growth-depressing action of threonine is obscure. Whether it represents a specific antagonism between nicotinic acid and the amino acid, or the result of the resolution of a multiple deficiency into one involving a single essential substance, is at present unknown. In regard to the former, one may consider the curious antagonism between threonine and muscle nicotinic acid as reported here.

SUMMARY

It was originally observed that an amino acid mixture containing lysine, valine, threonine, and histidine produced in rats on a low casein ration a growth retardation which was prevented by either supplementary nico-

tinic acid or tryptophan. The omission of lysine from the mixture did not significantly alter the results. Of the remaining components of the mixture, histidine and valine are without effect on growth. Threonine alone produces the growth retardation originally observed with the amino acid mixture. Supplementary nicotinic acid or tryptophan not only corrects the inhibition, but permits good growth, which is not possible in the absence of threonine.

The inhibition by threonine is not accompanied by a decrease in the storage of nicotinic acid in the liver or muscle. The values are usually in excess of those found on the basal diet alone. With nicotinic acid or tryptophan the vitamin storage in the liver is increased to a nearly normal level for the former and to a value considerably in excess of the normal for the latter supplement.

The authors are grateful to Mr. Horace Hayes for the care of animals employed in this investigation.

BIBLIOGRAPHY

1. Singal, S. A., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, **171**, 203 (1947).
2. Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109**, 657 (1935).
3. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
4. Cheldelin, V. H., Eppright, M. A., Snell, E. E., and Guirard, B. M., *Univ. Texas Pub., No. 4237*, 32 (1942).
5. Krehl, W. A., Teply, L. J., and Elvehjem, C. A., *Science*, **101**, 283 (1945).
6. Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A., *Science*, **101**, 489 (1945).
7. Briggs, G. M., *J. Biol. Chem.*, **161**, 749 (1945).
8. Briggs, G. M., Groschke, A. C., and Lillie, R. J., *J. Nutr.*, **32**, 659 (1946).
9. Groschke, A. C., and Briggs, G. M., *J. Biol. Chem.*, **165**, 739 (1946).
10. Groschke, A. C., Anderson, J. O., and Briggs, G. M., *Federation Proc.*, **6**, 257 (1947).
11. Krehl, W. A., Sarma, P. S., and Elvehjem, C. A., *J. Biol. Chem.*, **162**, 403 (1946).
12. Krehl, W. A., Henderson, L. M., de la Huerga, J., and Elvehjem, C. A., *J. Biol. Chem.*, **166**, 531 (1946).
13. Henderson, L. M., Deodhar, T., Krehl, W. A., and Elvehjem, C. A., *J. Biol. Chem.*, **170**, 261 (1947).
14. Hall, W. K., and Sydenstricker, V. P., *Arch. Biochem.*, **12**, 147 (1947).
15. Griffith, W. H., and Nawrocki, M. F., *Federation Proc.*, **7**, 288 (1948).
16. Salmon, W. D., *J. Nutr.*, **33**, 155 (1947).
17. Niven, C. F., Washburn, M. R., and Sperling, G. A., *Proc. Soc. Exp. Biol. and Med.*, **63**, 1 (1946).

THE NICOTINIC ACID CONTENT OF TISSUES OF RATS ON CORN RATIONS*

By S. A. SINGAL, V. P. SYDENSTRICKER, AND JULIA M. LITTLEJOHN

(From the Departments of Biochemistry and Medicine, University of Georgia School of Medicine, Augusta)

(Received for publication, July 23, 1948)

With the demonstration that nicotinic acid was a dietary essential for the dog (1), pig (2), monkey (3), chick (4), and man (5–9), it was rapidly shown that the nicotinic acid or pyridine nucleotide content of some tissues is markedly decreased in the deficient animal. Of the tissues studied only voluntary muscle and liver have shown subnormal values in the dog (10–14), pig (14), and chick (15). In pellagra the coenzyme content of muscle decreases as the deficiency progresses (16). Normal values are obtained for erythrocytes (13, 16, 17).

Prior to 1945 no specific disease due to nicotinic acid deficiency was demonstrable in the rat. There was evidence, however, that the rat could synthesize a sufficient amount of the vitamin for growth (18–20). In 1945 Krehl *et al.* (21, 22) observed in rats on low casein diets containing corn a growth retardation, which was prevented by either nicotinic acid or tryptophan. It seemed desirable, therefore, to compare the effects of nicotinic acid deficiency in the rat on the tissue content of this vitamin with those reported for other species. The results of this investigation are reported here.

EXPERIMENTAL

Wistar strain rats, 21 to 24 days old, were used in these experiments. The synthetic diet consisted of casein (Labco) 15, sucrose 76, cottonseed oil 3, salts (23) 4, cystine 0.5, and cod liver oil 2 parts. Vitamins were incorporated in 100 gm. of diet at the following levels: thiamine 2.0 mg., riboflavin 2.0 mg., pyridoxine 2.0 mg., calcium pantothenate 4.0 mg., and choline chloride 1.0 gm. The basal ration was prepared by mixing 60 parts of the above diet with 40 parts of corn grits.

Tissue specimens were prepared for analysis by autoclaving the finely ground material with 1.0 N H_2SO_4 for 30 minutes at 15 pounds. The hot extracts were neutralized to pH 4.5 with brom-cresol green as indicator,

---

* Acknowledgment is made of aid from the John and Mary R. Markle Foundation, and the United States Public Health Service. Crystalline vitamins and DL-tryptophan were generously contributed by Merck and Company, Inc., and Winthrop-Stearns, Inc., respectively.

cooled to room temperature, filtered, and neutralized to pH 6.8 with bromthymol blue as indicator. 1.0 ml. of blood obtained by cardiac puncture was laked with 50 ml. of distilled water and autoclaved with 5.0 ml. of 2.0 N H_2SO_4. From this point the preparation followed the procedure employed for the solid tissues. The nicotinic acid content of the extracts was determined microbiologically by the method of Snell and Wright (24).

Table I

Nicotinic Acid Content of Tissues of Rats on Corn Rations

Nicotinic acid content expressed as micrograms per gm. of wet tissue or micrograms per ml. of blood. Values in parentheses represent the range.

Diet 1, stock ration; Diet 2, basal; Diet 3, basal + 100 γ of nicotinic acid per rat daily; Diet 4, basal + 0.5 per cent L-tryptophan; Diet 5, basal + 200 γ of N^1-methylnicotinamide per rat daily.

	Diet 1, 7 rats	Diet 2, 12 rats	Diet 3, 13 rats	Diet 4, 11 rats	Diet 5, 12 rats
Weight change, *gm.*	+190	+26	+180	+192	+39
	(155–220)	(2–77)	(133–218)	(154–213)	(8–72)
Brain	47	30	48	50	
	(41–50)	(20–35)	(40–54)	(37–55)	
Heart	114	105	107	105	107
	(108–119)	(83–122)	(70–142)	(90–119)	(80–115)
Lung	48	43	46	50	
	(42–52)	(37–45)	(42–52)	(43–55)	
Spleen	58	57	61	63	
	(55–67)	(43–64)	(55–70)	(55–73)	
Muscle	78	45	77	81	40
	(73–84)	(16–60)	(55–97)	(70–92)	(18–74)
Kidney	87	84	101	101	84
	(80–92)	(66–104)	(81–130)	(80–119)	(61–94)
Liver	157	105	160	214	111
	(130–173)	(57–136)	(128–202)	(173–334)	(79–153)
Blood	14	12	13	14	13
	(12–18)	(11–13)	(12–14)	(13–17)	(11–14)

It is evident from the results in Table I that the effects of nicotinic acid deficiency in the rat resemble in part those observed in other species. After 70 days on the deficient diet, there is little change in the nicotinic acid content of heart, lung, spleen, kidney, and blood. Only in liver, voluntary muscle, and brain are subnormal values found. The changes in liver and muscle are similar to those reported for the rat by Hundley (25) and those for other species. It is surprising, however, to find a certain dependence of the nicotinic acid level of brain on the dietary level of the vitamin. By contrast, in the dog and pig (14) normal brain levels are maintained in spite of extreme deficiency. It appears that the major nicotinic acid stores in the rat are in the liver and muscle, when one considers not only the mass of tissue involved, but also its nicotinic acid content.

The administration of nicotinic acid completely prevents the changes in concentration of tissue vitamin which occur in animals on the basal ration alone. The values obtained are in general agreement with those found in animals reared on a stock ration which permits normal growth and breeding in this laboratory. With supplementary tryptophan essentially similar results are found. With tryptophan regarded as a biological precursor of nicotinic acid (26–28), the nature of its beneficial effect in maintenance of normal levels of tissue nicotinic acid is obvious. One finds levels in liver nicotinic acid which are above normal. Values as high as 334 γ per gm. are well outside the normal range. Similar results obtained since the completion of the present investigation have been reported (29) and interpreted as supporting evidence of the synthesis of nicotinic acid in the liver. As judged by the strict maintenance of normal values in other tissues, one might surmise that the liver is the major site of synthesis.

Najjar *et al.* (30) have reported that N^1-methylnicotinamide, a major metabolic end-product of nicotinic acid, possesses definite antiblacktongue activity, both as a preventive and as a therapeutic agent. This is in contrast to the findings of Teply *et al.* (31). In the present study this substance is inactive in rats at a daily level of 200 γ. It does not prevent either growth retardation or depletion of the nicotinic acid stores in the liver and muscle that is observed on the basal ration. It would appear that this species is unable to demethylate N^1-methylnicotinamide to nicotinic acid at a rate sufficient for good growth. In light of these results it is difficult to account for the lipotropic activity of N^1-methylnicotinamide in rats on diets containing glycocyamine, as reported by Najjar (32). If labile methyl groups are made available in a demethylation process, nicotinic acid *per se* cannot be considered an end-product, in view of the fact that the methylated derivative is inactive both as a preventive and as a therapeutic agent[1] in rats on the deficient basal ration.

DISCUSSION

The maintenance of the normal vitamin content of certain tissues in animals on diets deficient in this essential has been regarded as a necessary condition for the preservation of certain vital functions. A decrease is considered incompatible with life. In this respect the rat maintains normal nicotinic acid levels in the heart, lung, spleen, kidney, and blood, but allows depletion of vitamin stores in muscle and liver, and, to a smaller extent, in brain. The depressed values must reflect a diminished capacity for metabolic functions requiring the presence of nicotinic acid as a coenzyme. However, the rat on inadequate diets makes a nutritional adaptation by reducing the food intake and thereby lessening the effects of nutritional stress. When this curtailment of food intake is prevented by forced feeding, acute deficiency states are obtained in a relatively short time without the complication of a generalized undernutrition. An excellent example of this has been reported recently by Spector in the production of tryptophan deficiency in the rat (33).

---

[1] 1.0 mg. of N^1-methylnicotinamide was injected each day for 5 days into nicotinic acid-deficient rats. No alteration in the growth rate was observed during the next 14 days.

SUMMARY

The nicotinic acid content has been determined in some tissues of rats on diets containing corn, deficient in nicotinic acid. In the deficiency state normal values are maintained in the heart, lung, spleen, kidney, and blood. In the liver, muscle, and brain subnormal levels are obtained.

With supplementary nicotinic acid or tryptophan the depletion of the nicotinic acid stores is prevented. In this respect the beneficial action of the amino acid is interpreted in the light of the biosynthesis of nicotinic acid.

N^1-Methylnicotinamide does not prevent either the growth retardation or the depletion of nicotinic acid stores in the liver and muscle that is observed in rats on the deficient diet.

BIBLIOGRAPHY

1. Elvehjem, C. A. , Madden, R. J., Strong, F. M., and Woolley, D. W., *J. Biol. Chem.*, **123**, 137 (1938).
2. Chick, H., Macrae, T. F., Martin, A. J. P., and Martin, C. J., *Biochem. J.*, **32**, 10 (1938).
3. Harris, L. J., *Biochem. J.*, **32**, 1479 (1938).
4. Briggs, G. M., Jr., Luckey, R. D., Teply, L. J., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **148**, 517 (1943).
5. Smith, D. T., Ruffin, J. M., and Smith, S. G., *J. Am. Med. Assn.*, **109**, 2054 (1937).
6. Fouts, P. J., Helmer, O. M., Lepkovsky, S., and Jukes, T. H., *Proc. Soc. Exp. Biol. and Med.*, **37**, 405 (1937).
7. Spies, T. D., Cooper, C., and Blankenhorn, M. A., *J. Am. Med. Assn.*, **110**, 622 (1938).
8. Harris, L., and Hassan, A., *Lancet*, **2**, 1467 (1937).
9. Sydenstricker, V. P., Schmidt, H. L., Jr., Fulton, M. C., New, J. S., and Geeslin, L. E., *Southern Med. J.*, **31**, 1155 (1938).
10. Kohn, H. I., Klein, J. R., and Dann, W. J., *Biochem. J.*, **33**, 1432 (1939).
11. Pittman, M., and Fraser, H. F., *Pub. Health Rep., U. S. P. H. S.*, **55**, 915 (1940).
12. Dann, W. J., and Handler, P., *J. Nutr.*, **22**, 409 (1941).
13. Klein, J. R., Perlzweig, W. A., and Handler, P., *J. Biol. Chem.*, **145**, 27 (1942).

14. Axelrod, A. E., Madden, R. J., and Elvehjem, C. A., *J. Biol. Chem.*, **131,** 85 (1939).
15. Anderson, E. G., Teply, L. J., and Elvehjem, C. A., *Arch. Biochem.*, **3,** 357 (1943–44).
16. Axelrod, A. E., Spies, T. D., and Elvehjem, C. A., *J. Biol. Chem.*, **138,** 667 (1941).
17. Kohn, H. I., and Bernheim, F., *J. Clin. Invest.*, **18,** 585 (1939).
18. Dann, W. J., and Kohn, H. I., *J. Biol. Chem.*, **136,** 435 (1940).
19. Dann, W. J., *J. Biol. Chem.*, **141,** 803 (1941).
20. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **142,** 401 (1942).
21. Krehl, W. A., Teply, L. J., and Elvehjem, C. A., *Science*, **101,** 283 (1945).
22. Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A., *Science*, **101,** 489 (1945).
23. Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109,** 657 (1935).
24. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139,** 675 (1941).
25. Hundley, J. M., *J. Nutr.*, **34,** 253 (1947).
26. Rosen, F., Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **163,** 343 (1946).
27. Krehl, W. A., de la Huerga, J., and Elvehjem, C. A., *J. Biol. Chem.*, **164,** 551 (1946).
28. Singal, S. A., Briggs, A. P., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, **166,** 573 (1946).
29. Singal, S. A., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, **171,** 203 (1947).
30. Najjar, V. A., Hammond, M. H., English, M. A., Wooden, M. B., and Deal, C. C., *Bull. Johns Hopkins Hosp.*, **74,** 406 (1944).
31. Teply, L. J., Krehl, W. A., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **58,** 169 (1945).
32. Najjar, V. A., and Deal, C. C., *J. Biol. Chem.*, **162,** 741 (1946).
33. Spector, H., *J. Biol. Chem.*, **173,** 659 (1948).

THE ASSAY OF ANIMAL TISSUES FOR RESPIRATORY ENZYMES

VIII. CONDITIONS FOR OXALACETATE OXIDATION IN WHOLE HOMOGENATES*

By VAN R. POTTER, ARTHUR B. PARDEE,† AND GLORIA G. LYLE

(*From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison*)

(Received for publication, July 17, 1948)

In the development of the conditions for oxalacetate oxidation in animal tissues two sets of variables had to be studied. In the paper by Potter, LePage, and Klug (1) the homogenate was the chief variable. It was shown that isotonic KCl homogenates were superior to water homogenates, that the enzyme was stable in KCl homogenates at 0° for 6 hours or in the animal for 30 minutes post mortem, and that the enzyme was rapidly inactivated in the absence of substrate and cofactors at 38°. The present paper will report the results of studies in which the reaction mixture was varied in an attempt to define the optimum conditions for the oxidation, which are approximately the same as those previously used (1). The importance of this work lies in the fact that this reaction mixture can be used as a basis not only for studying the oxalacetate oxidation *per se* but also for studies on endergonic reactions which must be coupled to adenosine triphosphate (ATP). It must be emphasized, however, that the conditions described below are not necessarily the optimum for endergonic syntheses but are to be considered as a basis for further development of such reactions.

EXPERIMENTAL

Methods—Oxygen uptake was taken as a measure of enzyme activity, and the measurements were made in a conventional Warburg apparatus at 38°. In addition, it was frequently desirable to determine the rate of substrate disappearance and of product formation. This was done by stopping the reaction in appropriate flasks at various intervals by adding 2 ml. of cold 17.5 per cent trichloroacetic acid. The precipitated proteins were then centrifuged in the cold, and aliquots of the supernatants were analyzed.

The homogenates were always prepared with 9 volumes of isotonic KCl

---

* This work was aided in part by a grant from the Jonathan Bowman Fund for Cancer Research.

† Merck Postdoctoral Fellow in the Natural Sciences under the National Research Council.

per weight of tissue, with the precautions as to speed and cold previously indicated (1). Cold homogenates were added to cold reaction mixtures, which were then placed in the 38° bath. The final volume was always 3.0 ml. Readings were taken every 10 minutes, following a 10 minute equilibration period.

The analytical work herein reported involved mainly the disappearance of oxalacetate and the formation of citrate, although in experiments not reported determinations for α-ketoglutarate, succinate, and malate were carried out and these substances were shown to be formed in the few reactions examined. Oxalacetate gives the same reaction with 2,4-dinitrophenylhydrazine that is given by pyruvate when the treatment with the reagent is continued for 20 minutes in the direct method of Friedemann and Haugen (2). Interference due to α-ketoglutarate can be corrected by determining the optical density at two wave-lengths (480 and 580 mμ), but in most of the work this was not necessary. Since oxalacetate and pyruvate give the same color density, the determination of keto acid disappearance reveals the conversion of oxalacetate and pyruvate into other compounds and does not show any interconversion that may occur between the two compounds. The primary product of the oxidative reaction that results in oxalacetic disappearance is not known, but the formation of citrate would constitute strong evidence that the Krebs condensation had occurred. This substance was determined by the pentabromoacetone method as modified by Natelson *et al.* (3), with single extractions suggested by Lardy (4). The method may be further simplified by carrying out the reactions and extractions in open 50 ml. graduated centrifuge tubes. The extractions can be completed in about 1 minute by means of a high speed stirrer in place of shaking. Aliquots containing 10 to 60 γ of citric acid are suitable (3).

Optimum Concentrations—Table I summarizes a number of experiments in which varying concentrations of ATP, oxalacetate, $MgCl_2$, inorganic phosphate, and KCl were individually tested in the presence of the optimum concentrations of all the other reactants. Homogenates of rat kidney and rat liver were tested and gave approximately the same optima. Cytochrome *c* was present in all the flasks at a concentration of 1.3×10^{-5} M, although the beneficial effect was very slight and the compound could probably be omitted for most purposes. For example, in another set of experiments, the omission of the cytochrome decreased the oxygen uptake from 125 to 118 microliters per 20 minutes in the case of kidney and from 89 to 76 microliters in the case of liver.

All of the experiments in Table I were carried out with K salts instead of Na salts. In separate experiments it was found that the use of K salts produced a very marked stimulation in the case of kidney, while the effect in the case of liver, though slight, was still significant. Thus with kidney homogenate, the average of two experiments done in triplicate was 126 microliters per 20 minutes with K salts and only 71 microliters with Na salts. With liver the values were 114 and 101 microliters respectively.

TABLE I

Optimum Conditions for Oxalacetic Oxidase System in Liver and Kidney Homogenates

The source of enzyme was rat tissue; 0.3 ml. of kidney or 0.5 ml. of liver as a 10 per cent homogenate in isotonic KCl was added to each flask. The data for each compound were obtained from two or more experiments in duplicate, with freshly prepared homogenates. Thus the variation in animals is the source of the variation in plateau values obtained with various compounds. Each compound was studied at varying concentrations and all other compounds were held constant *and* at the optimum values. In addition to materials in the table 1.3 × 10^{-5} M cytochrome *c* was present in each flask. The concentration of each component selected for the standard technique is indicated by bold-faced type. The measure of activity was arbitrarily taken as the microliters of oxygen taken up in the 20 minutes following the 10 minute equilibration period.

ATP		Oxalacetate		$MgCl_2$		Phosphate		KCl*	
Molarity × 10^4	O_2	Molarity × 10^3	O_2	Molarity × 10^3	O_2	Molarity × 10^3	O_2	Molarity × 10^2	O_2
Kidney									
0	13	0	12	0	57	0	59	0	39
3.3	61	0.9	61	**3.3**	**132**	1.7	100	3.3	115
6.7	112	1.8	120	3.3	129	**3.3**	**103**	**6.7**	**130**
10.0	**124**	**2.7**	**122**	5.0	129	6.7	116	10.0	132
16.7	126	4.4	97	6.7	125	10.0	120		
						16.7	120		
Liver									
0	32	0	41	0	60	0	68	0	35
3.3	65	0.9	58	**1.7**	**82**	1.7	74	3.3	91
6.7	75	1.8	85	3.3	74	**3.3**	**71**	**6.7**	**81**
10.0	**80**	**2.7**	**85**	5.0	80	6.7	72	13.3	60
16.7	90	4.4	86	6.7	78	10.0	75	26.7	61
						16.7	80		

* Does not include KCl in the homogenate.

The data in Table I were obtained with reaction mixtures of approximately pH 7.0. Earlier work was done at about pH 7.4. The effect of pH on the oxygen uptake is shown in Fig. 1. The optimum pH for the system is not a property of any one enzyme, but represents the resultant of the various optima of different enzymatic components of the system. This fact probably explains the difference between the pH curve for liver compared to kidney, in which the ratio of the various enzymes involved is considerably different.

None of the data in Table I were obtained with mixtures that included DPN (diphosphopyridine nucleotide, coenzyme I). A large number of experiments were carried out on a series of ATP levels with and without added DPN. The DPN was of high purity (about 80 per cent), obtained from Dr. G. A. LePage. The experiments showed that at low levels of ATP DPN produced marked stimulation, but, as the level of ATP was increased, the effect of DPN disappeared. It appears that DPN is not needed *per se* but that it can substitute for ATP, particularly in a system that contains a limiting amount of ATP. These results can be interpreted more readily in view of the recent results of Kornberg and Lindberg (5) who reported that an enzyme from kidney splits DPN to adenylic acid and

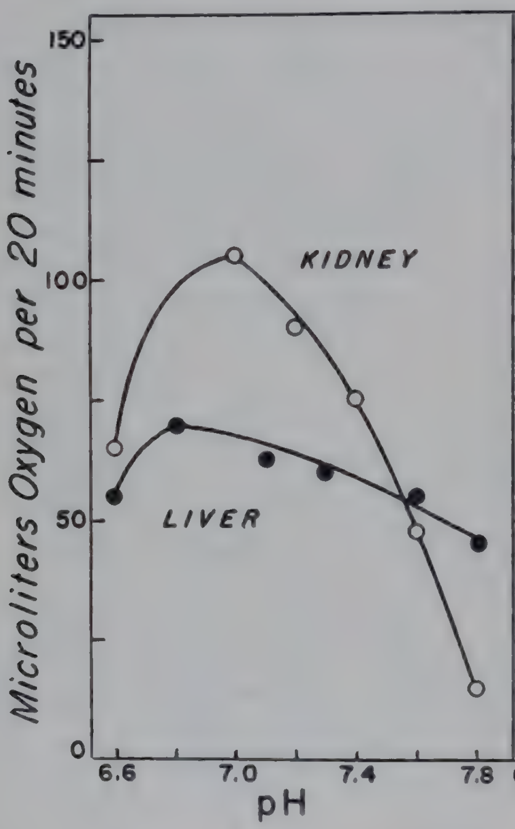


FIG. 1

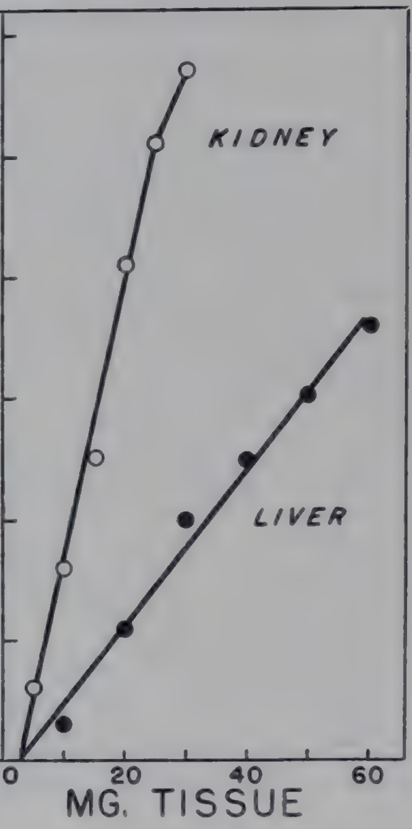


FIG. 2

FIG. 1. Effect of pH on oxalacetate oxidation by isotonic KCl homogenates of rat kidney (30 mg.) or liver (50 mg.). The reaction mixture was as described in the text except that the phosphate buffer was increased to 0.033 M. The data are averages of two experiments in duplicate. The pH values were essentially constant during the experimental period.

FIG. 2. Effect of tissue concentration on rate of oxygen uptake in the oxalacetate system, as described in the text.

nicotinamide ribose phosphate. Since adenylic acid can be phosphorylated to ATP in the reaction mixture we employ, the effect of DPN is probably merely to serve as a source of ATP. The new DPN-splitting enzyme is not inhibited by nicotinamide (5), and we have been unable to obtain any beneficial effects with nicotinamide in the oxalacetate system in kidney and liver homogenates. In contrast to the kidney enzyme the DPNase in brain and in tumor is inhibited by nicotinamide, and no doubt each tissue will have to be studied individually to determine the effect of various additions on the system.

The data in Table I show that the addition of inorganic phosphate produces a marked stimulation and that high amounts of phosphate are not harmful. The higher levels of phosphate give much better buffering capacity, but, if changes in the phosphate concentration due to oxidative phosphorylation are to be studied, the experimental error becomes too high to permit accurate determination of small changes. The level of phosphate to be used is therefore a compromise between these two considerations and is generally at the high level when changes in the level are not being studied. The control of the pH of the reaction is one of the problems not fully solved. The initial addition of oxalacetate lowers the pH because the oxalacetic acid is only three-fourths neutralized. Large amounts of this oxalacetate can lower the pH to a damaging level. However, the decarboxylation to pyruvate results in the loss of 1 acid equivalent, and causes an alkaline shift if fully neutralized oxalacetate is used. High levels of tissue can tolerate high levels of the oxalacetate much better than lower levels of tissue because of the more rapid removal of acid equivalents.

An examination of the data in Table I shows that the following levels of reactants are satisfactory for both kidney and liver: 0.067 M KCl, 3.3×10^{-3} M $MgCl_2$, 3.3×10^{-3} M potassium phosphate, 1.3×10^{-5} M cytochrome *c*, 2.7×10^{-3} M oxalacetate, and 1×10^{-3} M ATP (K salt).[1] The first four components can be combined into a stock solution of which 1.5 ml. are added per flask. The solution is prepared by adding 1.49 gm. of KCl, 0.203 gm. of $MgCl_2 \cdot 6H_2O$, 0.136 gm. of KH_2PO_4, and 10 ml. of 4×10^{-4} M cytochrome *c* to 140 ml. of water, adjusting the pH to 7.0 with KOH, and making up to a final volume of 150 ml.

In addition to the 1.5 ml. of stock solution per flask, water is added to make the *final* volume 3.0 ml., followed by 0.3 ml. of 0.01 M ATP, 0.3 ml. of 0.0267 M fresh oxalacetate, and 0.2 to 0.7 ml. of 10 per cent KCl homogenate. All components were kept cold. The center wells were prepared before the oxalacetate addition, with 0.2 ml. of 2 N NaOH and filter paper. Table I shows that the addition of considerable amounts of extra KCl is without effect, and in general the variations in the amount of homogenate or addition of moderate amounts of extra substrates or inhibitors were compensated by merely adjusting the volume of the water added.[2]

---

[1] A commercial sample of sodium ATP (Rohm and Haas, Philadelphia) was found to be unsatisfactory in this system unless given further purification designed to remove traces of metallic inhibitors. The material as received yielded about 50 per cent activity compared with ATP prepared by Dr. G. A. LePage.

Proportionality—One of the requirements of an assay system is that the amount of oxygen uptake is proportional to the amount of tissue added. The conditions described above permit oxygen uptakes within the range of convenient manometric measurement that are proportional to the amount of either kidney or liver homogenate, as shown in Fig. 2. With higher levels of oxalacetate such data are not obtained, and the deviation is especially marked at the lower levels of tissue. The data in Fig. 2 show a slight displacement to the right, which may be related to the oxalacetate effect or may be due to traces of some metal contaminant, although extreme precautions are taken to avoid metals.

Nature of Reaction—It is still not possible to accept the oxygen uptake data as an assay of a single enzyme. It appears that the oxalacetate is oxidized via the Krebs cycle, and in the case of liver and kidney appreciable amounts of citrate are formed. If the amounts of keto acid disappearing are plotted against time, it is seen that there is a rapid disappearance of keto acid, together with a rapid rise in citrate and malate. The reaction can also be analyzed by examining the effect of varying concentrations of the substrate upon substrate disappearance, oxygen uptake, and citrate formation, as shown in Fig. 3. Six different tissues, including two types of tumors,[3] were studied on this basis. The incubation time was 40 minutes and was chosen on the basis of the experiments referred to above. Although malate was not determined in this series of experiments, the data show that increasing amounts of oxalacetate result in a diminution of the oxygen taken up, while the amount of keto acid disappearing increases. This indicates that with increasing amounts of oxalacetate the reduction of oxalacetate to malate assumes increasing importance and a dismutation of oxalacetate to malate and citrate begins to supplant the oxidative conversion of oxalacetate to citrate. These results with extremely small quantities of rat tissue fortified with ATP are thus in harmony with the older data in the literature, in which minces of pigeon breast muscle were used by Krebs (see (7)). They are also compatible with the results of Kalnitsky (8), who has used high concentrations of oxalacetate in the study of citrate formation. It is interesting to note that negligible quantities of citrate were formed by brain and heart homogenates, although keto acid disappeared and oxygen was taken up. The citrate formation in the charts is of course the resultant of the rates of formation and utilization. Thus the lack of citrate formation in the case of low levels of oxalacetate in kidney and liver is due to the fact that the citrate formation ceased when the oxalacetate was used up, and the accumulated citrate disappeared. The results with the tumor tissues support the earlier findings and show in addition that not only is the oxygen uptake very low but that the keto acid disappearance is very low also.

---

[2] The function of the KCl appears to be mainly the regulation of the tonicity of the solution. A number of experiments were done with sucrose in place of KCl in view of recent studies with this compound (6). Isotonic sucrose homogenates (8.5 per cent sucrose) were superior to 30 per cent sucrose homogenates, although the latter appear to preserve the mitochondria better (6). The optimum final sucrose concentration was between 2.5 and 5.5 per cent. In 4.5 per cent sucrose the reaction was independent of KCl concentration up to 6 mg. of KCl per ml. Apparently the requirement for K ion is met by the other K salts in the medium. Sucrose homogenates showed no advantage over KCl homogenates in the oxidation of oxalacetate, although other possible advantages are not ruled out.

[3] We are indebted to Mr. B. E. Kline for supplying us with rats bearing the Flexner-Jobling and the Walker No. 256 transplantable tumors.

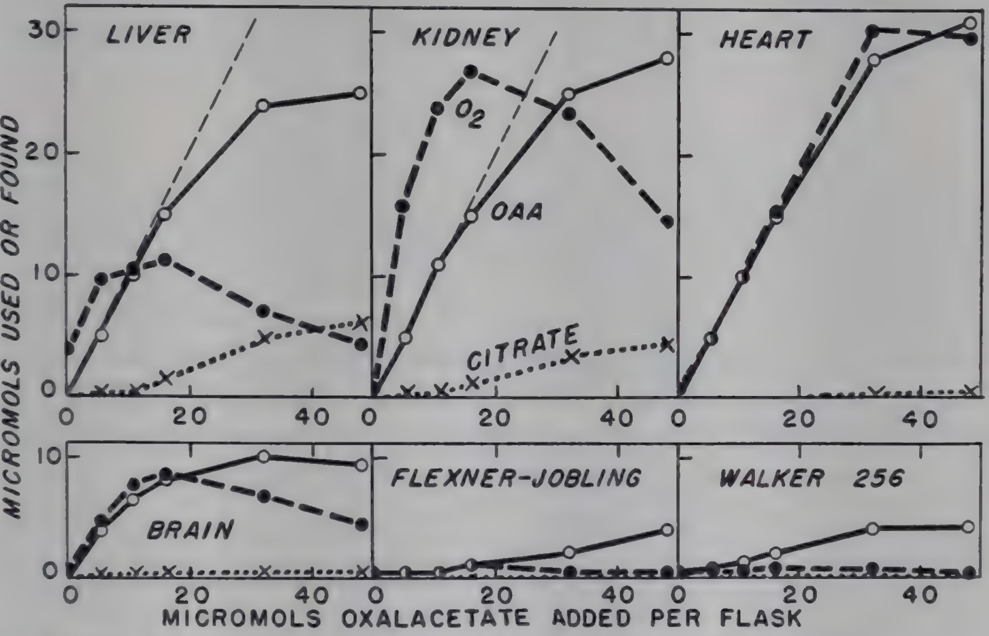


FIG. 3. Effect of oxalacetate concentration on oxygen uptake (O_2), oxalacetate utilization (OAA), and citrate formation in the standard oxalacetate system but with 0.0167 M phosphate buffer. Tumors and normal tissues from rats (50 mg. per flask). The labels on the curves for kidney apply to the other tissues as well. The light dashed line in the case of liver and kidney gives the theoretical curve for 100 per cent removal of oxalacetate, and applies to all of the tissues.

The above results suggest that the oxygen uptake during the initial part of the reaction may be a measure of that portion of the Krebs cycle which is most closely connected with the oxidative phase of oxalacetate removal; hence our use of the term "oxalacetic oxidase." It is clear that the oxygen uptake very rapidly becomes the sum of several oxidative steps occurring in other parts of the cycle. Furthermore, the occurrence of alternative metabolic pathways is not excluded. For example, oxalacetate to pyruvate to acetate to acetoacetate could occur. However, the presence of oxalacetate tends to deflect this pathway into the Krebs cycle, as Lehninger has shown (9).

In view of the probability of other reactions in the cycle contributing to the oxygen uptake, it was of interest to determine the rates of oxidation for some of the compounds that might be involved. This was done with four different tissues by use of pyruvate, citrate, α-ketoglutarate, succinate, malate, no substrate, and oxalacetate for comparison. The results are shown in Table II. In each case the data are given in comparison with oxalacetate taken as 100 for the given tissue, but the absolute value for oxalacetate is also given. In heart homogenate, pyruvate was superior to oxalacetate, but in the other tissues it was not as actively oxidized. Similarly α-ketoglutarate was rapidly utilized by heart. In contrast, citrate was very poorly utilized by heart, brain, and kidney in comparison with oxalacetate. Malate was also poorly utilized in comparison with oxalacetate. Thus there is little reason to believe that any member of the Krebs cycle could be substituted for oxalacetate in this system without substantially changing the results. In the data given in Table II, the

TABLE II

Relative Rates of Oxidation of Substrates

The source of enzyme was rat tissue; 0.3 ml. of kidney, 0.5 ml. of liver, 0.4 ml. of heart, or 0.7 ml. of brain as a 10 per cent homogenate in isotonic KCl was added to each flask. The standard reaction mixture was used, except that phosphate was 16.7 × 10^{-3} M and the substrates were 3.3 × 10^{-3} M. The oxygen uptake in the 20 minutes following the 10 minute equilibration was taken as 100 for oxalacetate and the uptakes for other substrates are given relative to this value. The actual rates for oxalacetate are given as Q_{O_2}. The results are averages of duplicates on each of two animals generally.

	Kidney	Q_{O_2}	Liver	Q_{O_2}	Heart	Q_{O_2}	Brain	Q_{O_2}
Oxalacetate	100	52.0	100	16.1	100	25.5	100	15.0
Pyruvate	59		79		123		83	
Citrate	39		85		19		27	
α-Ketoglutarate	97		92		156		81	
Succinate	65		80		71		68	
Malate	55		75		46		41	
No substrate	8		44		2		32	

substrates were used at the same level as oxalacetate. In the assay, they would be present at much lower levels. The data in Table II do not represent the maximum rates for the other substrates by any means, while the rates for oxalacetate represent the maximum that we have been able to attain. Succinate could be oxidized much more rapidly by adding calcium and increasing the succinate concentration (10). Malate could be oxidized more rapidly by adding DPN and glutamate (11). The test system therefore applies chiefly to oxalacetate. It may be noted that a mixture of oxalacetate and pyruvate is no better than oxalacetate for experiments such as those described in this paper, although the mixture may offer some advantages in that larger amounts may be used with less alteration in the acid-base balance.[4]

DISCUSSION

The conditions for maximum oxygen uptake in the oxalacetate system involve the use of a very low substrate concentration and the reaction soon fails for lack of substrate. The data suggest that the optimum substrate concentration is very low and in normal tissue *in situ* the oxalacetate concentration is probably no higher than this at the most. However, *in situ* the oxalacetate and pyruvate are continually being replenished and in addition the oxalacetate is in equilibrium with pyruvate and CO_2 by means of the Wood-Werkman reaction. In the Warburg flask, the use of the CO_2 absorbers in the center well cuts down the chances for this reaction to operate and limits the supply of oxalacetate to the original amount plus what can be formed from malate. Since the oxalacetate becomes depleted by decarboxylation, the studies *in vitro* are hampered by an inability to keep up the supply of oxalacetate. Possibly the reaction would benefit by the use of bicarbonate buffer and CO_2 in the gas phase (*cf.* Green *et al.* (12)).

The measurement of the oxygen uptake with the optimum level of oxalacetate for maximum rate is probably a fair measure of the oxidative component of the reactions involved in the formation of citrate, but there must be some reservations about any studies that involve oxygen uptake alone, and it is desirable to make analyses for as many products as possible. The complete lack of citrate accumulation in heart and brain raises the question of whether or not it was formed, and it would be of considerable interest to know whether acetate was formed to any extent in these tissues or in the tumor tissues. The observations on tumor tissues are of interest in view of the earlier findings of Elliott and Greig (13). Using tumor slices treated with malonate, they found no conversion of pyruvate to succinate, although some keto acid disappearance was noted, and acetic acid was tentatively identified.

---

[4] While the mixture of pyruvate and oxalacetate is no better than oxalacetate for short experiments with liver and kidney as reported in this paper, the mixture gives superior results with heart and brain, and, if pyruvate is present, other 4-carbondicarboxylic acids give results that compare favorably with those obtained with oxalacetate only. With oxalacetate alone, an optimum level of oxalacetate *and* pyruvate appears to be rapidly attained in liver and kidney but not in heart or brain homogenates. (Added in proof.)

SUMMARY

1. Optimum conditions for the study of the oxidation of oxalacetic acid by isotonic KCl homogenates of rat liver and kidney were described.

2. The final concentrations of reactants in the optimum system were 0.067 M KCl, 3.3×10^{-3} M $MgCl_2$, 3.3×10^{-3} M potassium phosphate, 1.3×10^{-5} M cytochrome *c*, 2.7×10^{-3} M oxalacetate, and 1×10^{-3} M ATP. From 0.2 to 0.7 ml. of 10 per cent homogenate could be used, depending on the tissue being examined. The final volume was 3.0 ml.

3. The rate of oxygen uptake was proportional to the tissue concentration, within the range of convenient manometric measurement.

4. The optimum pH was about 7.0, and the use of potassium salts gave better results than when all sodium salts were used.

5. Analyses for citrate accumulation showed that this compound was formed when liver and kidney were used, but when brain, heart, and tumor were studied, the citrate accumulation was negligible. However, in brain and heart there were appreciable oxygen uptake and keto acid disappearance, while with the tumors (Flexner-Jobling and Walker No. 256 rat, transplantable tumors) oxygen uptake and keto acid disappearance were both very low.

BIBLIOGRAPHY

1. Potter, V. R., LePage, G. A., and Klug, H. L., *J. Biol. Chem.*, **175**, 619 (1948).
2. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943).
3. Natelson, S., Lugovoy, J. K., and Pincus, J. B., *J. Biol. Chem.*, **170**, 597 (1947).
4. Lardy, H. A., in Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis (1945).
5. Kornberg, A., and Lindberg, O., *Federation Proc.*, **7**, 165 (1948).
6. Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *Proc. Soc. Exp. Biol. and Med.*, **65**, 320 (1947).
7. Krebs, H. A., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **3**, 191 (1943).
8. Kalnitsky, G., *Federation Proc.*, **7**, 162 (1948).
9. Lehninger, A. L., *J. Biol. Chem.*, **161**, 413 (1945).
10. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, **149**, 217 (1943).
11. Potter, V. R., *J. Biol. Chem.*, **165**, 311 (1946).
12. Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, **172**, 389 (1948).
13. Elliott, K. A. C., and Greig, M. E., *Biochem. J.*, **31**, 1021 (1937).

INHIBITION OF SUCCINIC DEHYDROGENASE BY OXALACETATE*

By ARTHUR B. PARDEE† and VAN R. POTTER

With the Technical Assistance of Gloria G. Lyle

(*From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison*)

(Received for publication, July 17, 1948)

Although the inhibition of succinic dehydrogenase by oxalacetate is a generally accepted fact, there is a paucity of data on the subject. In 1937 Das (1), using a modified Thunberg technique, reported that the enzyme was 50 per cent inhibited by 2×10^{-5} M oxalacetate when the succinate concentration was 0.025 M. In 1939 Potter (2) reported that the oxidation of succinate by liver and kidney homogenates was inhibited by cozymase (DPN). Keilin and Hartree (3) and Mann and Quastel (4) attributed this effect, no doubt correctly, to the formation of oxalacetate, although no data on the effect of oxalacetate were presented. The inhibitory effect of DPN upon the succinate system was later studied by Swingle, Axelrod, and Elvehjem (5) who also determined the effect of oxalacetate upon the succinic dehydrogenase system by measuring oxygen uptake. They reported that at succinate concentrations of 0.045 M oxalacetate produced 98, 65, and 22 per cent inhibition at concentrations of 50, 10, and 5×10^{-5} M. From the data given it is not possible to tell whether the inhibition was transitory, as will be shown below, or whether the experiments were of such short duration that the decreased inhibition was not revealed. Since we found that the inhibition declined with time, it is clear that the earlier experiments (1, 5) cannot be accepted as quantitative measures of the inhibition by oxalacetate. They do, however, establish the fact that this substance has a remarkable affinity for the succinic enzyme; remarkable because it appears to be at least 1000 times greater than the affinity of the normal substrate for the enzyme, and because oxalacetate has been assumed to be formed in the course of succinate oxidation. If the physiologically formed oxalacetate were as toxic to succinate oxidation as added oxalacetate, the inhibition would have profound regulatory effects upon oxidative metabolism. Such does not seem to be the case, however, although the reasons are as yet obscure.

---

* This work has been supported in part by the Jonathan Bowman Fund for Cancer Research.

† Merck Postdoctoral Fellow in the Natural Sciences under the National Research Council.

EXPERIMENTAL

Reactions were carried out in Warburg flasks at 38°. To each flask were added at 0°, in the order given, water to make the final volume 3.0 ml., 0.1 ml. of 1 M K phosphate buffer at pH 7.4, 0.1 ml. of 4×10^{-4} M cytochrome *c*, various amounts of 10 per cent water or KCl homogenates of rat tissues, 0.1 ml. of a solution containing 0.012 M $CaCl_2$ and 0.012 M $AlCl_3$, and 0.3 ml. of 0.5 M sodium succinate essentially as in the succinoxidase assay system (6). In addition, various concentrations of freshly prepared oxalacetate and pyruvate were added in order to test their separate and combined effects upon the succinic system. The oxygen uptake was measured at 10 minute intervals after a 10 minute equilibration. Zero time was taken as the time when the flasks were placed in the 38° bath, which was immediately after the oxalacetate addition. Oxalacetate or pyruvate additions followed the succinate addition.

In reporting the data, the oxygen uptake for successive 10 minute periods is plotted against time at the mid-point of each time interval. Since in the succinate system the substrate is present in large excess in order to give the maximum velocity, this method of plotting the data gives the relative amount of the functioning succinic dehydrogenase throughout the course of the experiment.

Total keto acids were determined by the direct method of Friedemann and Haugen (7). In this procedure, oxalacetic and pyruvic acids give the same result when the incubation with the reagent is 20 minutes.

Later experiments were carried out with the succinate and oxalacetate added to the oxalacetate oxidizing system, which contains no Ca or Al, and has Mg and adenosine triphosphate (ATP) added (8). Under the conditions described for the succinoxidase system (6) the homogenate will not oxidize malate, oxalacetate, or pyruvate, and oxidizes succinate quantitatively to fumarate and malate.[1]

Inhibition by Oxalacetate—When succinate is added to the succinoxidase system, the rate slowly declines in a linear fashion, as previously described (9) and as shown in the top curve of Fig. 1. From the initial rates of five similar experiments with variable amounts of succinate the Michaelis-Menten constant (10) was determined for this system and the average value of 0.006 mole per liter was obtained for subsequent calculations. When various amounts of 0.001 M oxalacetate were added to the standard succinate system, the oxidation was strongly inhibited, but with the passage of time the inhibition diminished, so that the rate of the oxidation progressively increased and approached the declining control curve for succinate alone (Fig. 1). Analysis of the flask contents at the end of the experiment showed that the total keto acid was the same as at the beginning.

[1] Unpublished experiments.

In view of the fact that neither oxalacetic nor pyruvic acid is oxidized in this system plus the known fact that oxalacetate is both spontaneously and enzymatically decarboxylated to pyruvate, it is concluded that the release from inhibition is due to the decarboxylation of the oxalacetate to pyruvate.

It was found that oxalacetate produced 50 per cent inhibition at a final concentration of approximately 2×10^{-5} M based on the initial inhibition. At concentrations at which oxalacetate produces 50 to 100 per cent inhibition, pyruvate is without effect; so that conversion of oxalacetate to pyruvate would account for the release from the inhibition. Final concentrations of 0.1 M pyruvate or α-ketoglutarate gave 50 per cent inhibition, while malate and aspartate at similar concentrations had no effect. The effect of oxalacetate is unique in our experience with forty or 50 inhibitors of the succinic system (9), since no other inhibitor gave an immediate inhibition that decreased with time. Previous inhibitors have either acted at once and maintained the inhibition, like malonate, or have given progressively *greater* inhibition, like quinone. The behavior of oxalacetate, as shown in Fig. 1, indicates that oxalacetate forms a dissociable complex with succinic dehydrogenase, as do succinate and malonate.

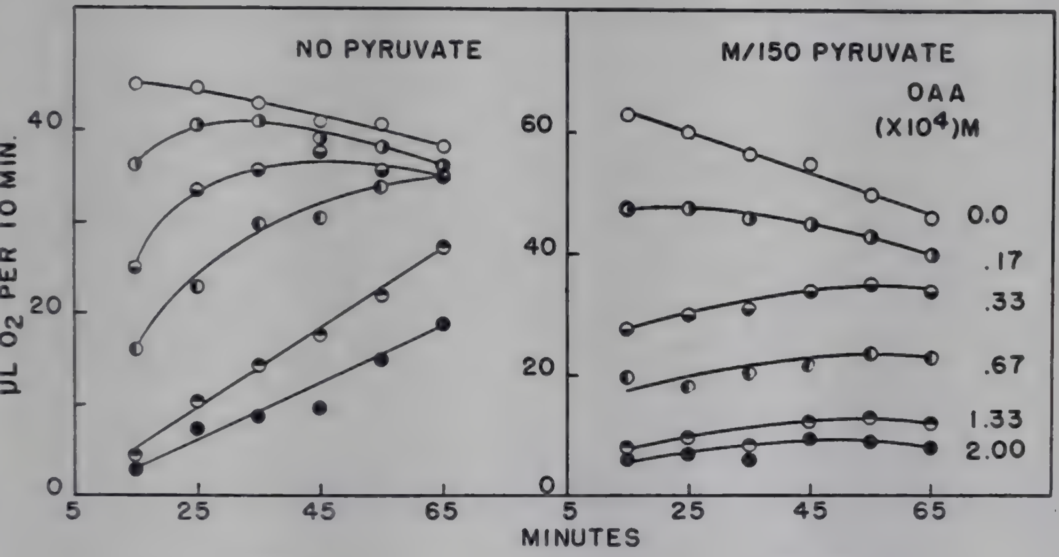


FIG. 1 FIG. 2

FIG. 1. Transitory inhibition of the succinoxidase system by various amounts of oxalacetate. Succinoxidase assay system described in the text, with 0.2 ml. of a 10 per cent water homogenate of rat liver per flask. Concentrations of oxalacetate as in Fig. 2, for the corresponding symbols.

FIG. 2. Stabilization of the inhibition of the succinoxidase system by oxalacetate (OAA) when pyruvate is present. Succinoxidase system as in Fig. 1. Oxalacetate concentrations refer to the final molarity.

Since both Ca and Al have been shown to increase the spontaneous decarboxylation of oxalacetate (11), experiments with either or both of those ions omitted were carried out, but the results were not affected very much and are complicated by the possibility of oxalacetate formation from succinate. On the other hand, when the amount of homogenate was varied, the rate at which the oxalacetate effect wore off was greatly increased, as will be shown later. This effect is attributed to the enzymatic decarboxylation of oxalacetate to pyruvate. Furthermore, the rate of release from oxalacetate inhibition was different in different tissues, which fell in the following order of decreasing rates: kidney, liver, heart, brain, and tumor (Walker No. 256 rat carcinoma). This property is probably a reflection of oxalacetate decarboxylation but was not studied extensively, and most of the experiments were carried out with rat liver.

Inhibition by Oxalacetate Plus Pyruvate—Since moderate concentrations of pyruvate do not inhibit succinate oxidation, it is possible to study the effect of pyruvate on the oxalacetate inhibition. It appears that at least with the low concentrations of oxalacetate used in these experiments pyruvate inhibits oxalacetate decarboxylation. In experiments in which various levels of oxalacetate were studied in the presence of M/150 pyruvate, the inhibition of the succinate system remained relatively constant throughout the experiment, showing a very slow rate of release (Fig. 2). These curves are in marked contrast to those in Fig. 1. The testing of the inhibition of the succinate system with and without pyruvate appears to be a highly specific test for oxalacetate, and is applicable to quantities of 10 to 50 γ. These amounts are far below the quantity required for methods that depend on CO_2 evolution. The test may be of some value when it is necessary to distinguish between oxalacetate and pyruvate.

Inhibition by Oxalacetate Produced from Succinate—In the assay method which has been proposed for the succinoxidase system (6), calcium and aluminum ions were added because of their demonstrated stimulatory effects, which were believed to be indirect. Swingle, Axelrod, and Elvehjem (5) had provided substantial evidence favoring the view that calcium is stimulatory because it accelerates the breakdown of DPN contained in the homogenates. It was postulated that the DPN in the homogenates would be sufficient, in the absence of calcium ions, to yield inhibitory amounts of oxalacetate. However, they did not determine the keto acids formed in the presence or absence of calcium in the succinoxidase system. Such a determination would test directly for the occurrence of the malate to oxalacetate step. We have now tested their idea by determining the effect of various additions to the succinoxidase system in terms of keto acid formed and oxygen taken up. The results of three such experiments with rat kidney homogenates are shown in Fig. 3. The same experiment was also carried out with liver with similar though less striking results.[2] The decreased oxygen uptake is clearly correlated with the formation of keto acid, which in view of the results in Figs. 1 and 2 must be a mixture of oxalacetic and pyruvic acids. For example 0.3 micromole of keto acid per flask is 1×10^{-4} M, which if 100 per cent oxalacetate would produce much more inhibition (Fig. 2) than was actually observed (Fig. 3).

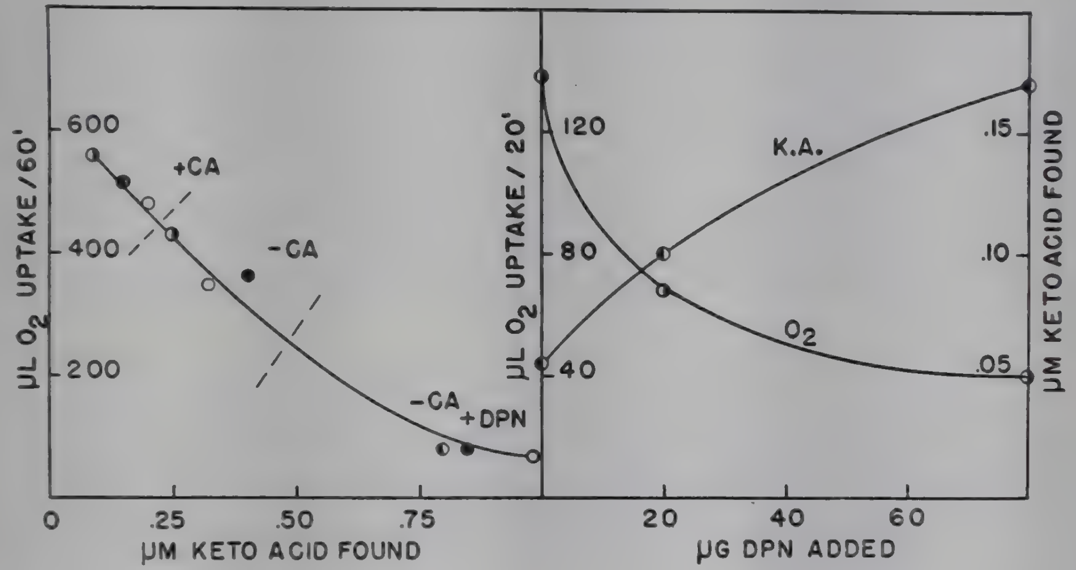


FIG. 3 FIG. 4

FIG. 3. Plot of oxygen uptake against keto acid formed in the succinoxidase system under various conditions to show that the formation of keto acid *decreases* the oxygen uptake. Experiments with three different rats represented by three different symbols. Each experiment was performed with 0.2 ml. of a 10 per cent cold isotonic KCl homogenate of rat kidney. Part of the curve, marked +Ca, represents conditions of standard succinoxidase assay (except for homogenate), the middle part of the curve, marked −Ca, represents the effect of omitting calcium, and the lower part of the curve, marked −Ca, +DPN, represents the effect of omitting calcium and adding DPN (approximately 400 γ).

FIG. 4. Effect of adding various amounts of DPN to the succinoxidase system in terms of both oxygen uptake and keto acid formation. Standard succinoxidase system with 0.1 ml. of a 10 per cent water homogenate of rat kidney.

The oxygen uptake was quite constant during the 60 minute period in the flasks in which Ca was omitted or DPN was added (Fig. 3), indicating that a steady state level of oxalacetate was rapidly reached. In other experiments with periodic sampling, the total keto acid increased with time. There was thus a continual production of oxalacetate that was maintained at a constant level by concomitant conversion to pyruvate.

---

[2] The data in Fig. 3 were obtained with KCl homogenates, but water homogenates gave almost identical results. With liver, however, the water homogenate did not give the same quantitative result that was obtained with the KCl homogenate. In all cases, decreased oxygen uptake was correlated with increased total keto acid.

The succinoxidase system is very sensitive to the presence of DPN because of the resultant oxalacetate that is formed: increased amounts of DPN (20 to 80 γ) give increased amounts of keto acid and decreased oxygen uptake (Fig. 4).

These results emphasize the importance of observing the recommended conditions for the succinoxidase assay system (6). Together with the results in Fig. 1, they show that the succinate to fumarate step can be isolated, and that, if small amounts of oxalacetate are formed initially, the resulting inhibition will disappear. Thus the maximum rate observed is the best measure of the succinoxidase system. In practice, the initial readings are seldom low.

Prevention of Oxalacetate Effect—In view of the failure to obtain maximum succinate oxidation in homogenates in the absence of calcium, it was always difficult to explain how Rosenthal (12) obtained the same rates of oxidation in rat liver slices that we obtained with homogenates. His results and the question of whether the oxalacetate concentration is a physiological regulator of succinate oxidation led us to test the effect of oxalacetate on succinate oxidation in a homogenate system that we regard as "physiological;" namely, the isotonic KCl homogenate, fortified with ATP and Mg (8). To our surprise, relatively large amounts of oxalacetate failed to inhibit succinate oxidation. In Table I it is shown that with the reaction mixture and the amount of oxalacetate that is optimum for the oxalacetate oxidation (8) oxidation proceeds vigorously when succinate is present with or without oxalacetate (132.6 and 117.5 microliters of O_2). On the other hand, when ATP is either omitted or added in the presence of calcium ions, which accelerate the dephosphorylation of ATP, the presence of oxalacetate prevents the oxidation of succinate. Thus oxalacetate inhibits succinate oxidation in the absence of ATP but not in its presence.

TABLE I

Prevention by ATP of Inhibition of Succinoxidase by Oxalacetate

Oxalacetate system as described by Potter, Pardee, and Lyle (8), with 0.2 ml. of a 10 per cent isotonic KCl homogenate of rat kidney plus 0.1 ml. of 0.5 M succinate, and, where indicated, calcium and aluminum as in the succinoxidase system described in the text. Oxygen uptake, microliters in 20 minutes; all measurements in duplicate; volume, 3.0 ml.

Reaction conditions	Oxalacetate added	
	None	0.00267 M
With 0.001 M ATP	117.5	132.6
ATP omitted	81.3	5.0
With ATP + calcium and aluminum	57.1	0

In other experiments it was possible to show that in a reaction mixture containing oxalacetate, ATP, and succinate delayed additions of calcium ions produced almost immediate inhibition of the succinoxidase system, the degree of inhibition depending upon the time of calcium addition. It was as if the oxalacetate were in a form that would not inhibit the enzyme and the addition of the calcium caused the oxalacetate to resume the inhibitory form. It may be emphasized that calcium *per se* does not inhibit the succinoxidase system. The question arises as to whether in the presence of ATP the oxalacetate might inhibit succinate oxidation but by being oxidized itself might obscure the succinate oxidation. In Table II various amounts of oxalacetate were added to a system containing ATP, with and without succinate. In the absence of succinate, the data show that the lower levels of oxalacetate were incapable of giving a rate of oxygen uptake that would account for the amount observed when succinate was also present. It must be concluded, therefore, that these levels of oxalacetate do not inhibit succinoxidase in the presence of ATP. Yet Figs. 1 and 2 showed that one-tenth as much oxalacetate inhibited the succinoxidase system in the absence of ATP.

TABLE II

Oxidation of Succinate in Presence of Oxalacetate and ATP

Oxalacetate system as described by Potter, Pardee, and Lyle (8), with 0.3 ml. of a 10 per cent isotonic KCl homogenate of rat kidney plus 0.3 ml. of 0.01 M ATP with various amounts of exactly neutralized oxalacetic acid with and without succinate. Volume, 3.0 ml.; oxygen uptake, microliters in 20 minutes.

Succinate	Final oxalacetate concentration ($\times 10^4$ M)					
	0	3.3	6.7	13.3	26.7	53.3
Present	213	215	205.8	210.5	196.6	197.6
Absent	6.9	44.7	65.5	84.6	109.5	84.4

DISCUSSION

Inhibition of Succinoxidase by Oxalacetate—The results can be accounted for by assuming that oxalacetate competitively inhibits the oxidation of succinate according to the equation

$$v = \frac{SV}{K_s(1 + (X/K_i)) + S}$$

where v is the rate of oxygen uptake, V is a constant, S is succinate concentration, K_s is the Michaelis-Menten constant, X is the oxalacetate (inhibitor) concentration, and K_i is the dissociation constant of oxalacetate and enzyme. Let H be the rate of O_2 uptake in the absence of X, and write the equation in the form

$$\frac{K_s X}{K_i(S + K_s)} = \frac{H}{v} - 1$$

If S is essentially constant during the measurements, as it is in these experiments, X is proportional to $(H/v) - 1$. If it is assumed that the rate of breakdown of oxalacetate is first order, $\log X_0/X = kt$, where X_0 is the concentration of X at zero time, a plot of $\log ((H/v) - 1)$ *versus* t should give a straight line of intercept at zero time,

$$\log \frac{K_s X_0}{K_i(S + K_s)}$$

and slope, $-k$.

Data from an experiment similar to that shown in Fig. 1, but with variable homogenate and constant oxalacetate, were plotted in this way and gave the results shown in Fig. 5. The intercept at zero time, which is proportional to the oxalacetate added (see below), is the same for all three concentrations of homogenate, showing that the initial inhibition is independent of the amount of homogenate. In the lower left corner of Fig. 5 the slopes of the three lines are plotted against the amount of homogenate and a straight line is obtained, showing that in addition to the slow spontaneous decarboxylation there was a decarboxylation that was proportional to the homogenate concentration.

With the same method of plotting the data, but with constant homogenate and variable oxalacetate, a series of lines having the same slope but different intercepts is obtained (Fig. 6). When the antilogs of the intercepts were plotted against the concentration of oxalacetate, the result was as shown in Fig. 7. The graph should be a straight line through the origin and the slight displacement to the right may be due to a small spontaneous decomposition before the beginning of the experiment.

The value for K_i can be calculated from Fig. 7, and is found to be 1.5×10^{-6} M. Thus oxalacetate is a much more powerful inhibitor than malonate, for which the constant is 10^{-4} M, calculated from Potter and DuBois (9).

ATP Effect—Although oxalacetate is a powerful inhibitor of the succinoxidase system as shown in Figs. 1 and 2, it appears that this effect can be prevented by the presence of ATP. Whether the ATP acts by shielding the succinoxidase directly or indirectly or whether the ATP converts the oxalacetate to a form that cannot combine with the succinic dehydrogenase cannot be decided at this time. It has been suggested by various investigators that a phosphooxalacetate may exist, but only indirect evidence

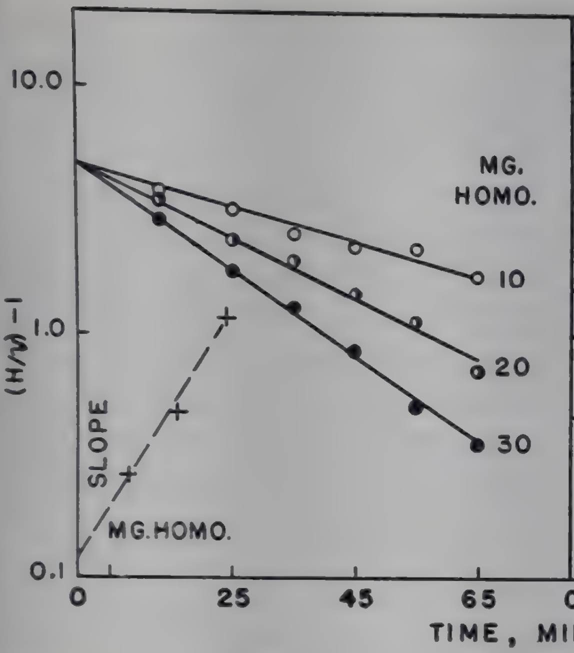


FIG. 5

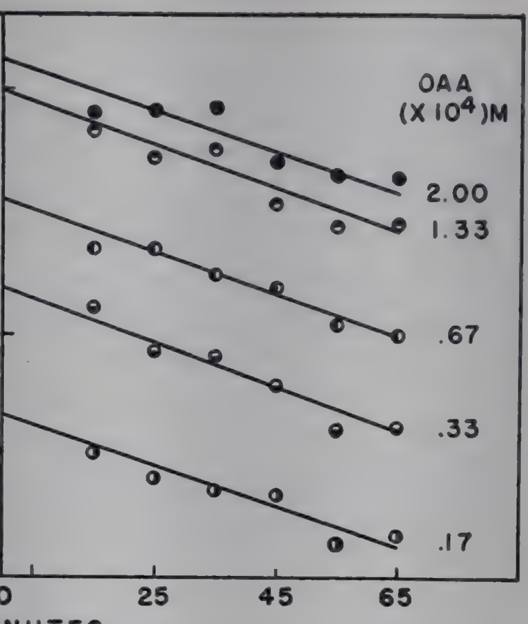


FIG. 6

FIG. 5. Log of $(H/v)-1$ plotted against the time of reaction for three amounts of liver homogenate as indicated. Succinoxidase system as in the text. Initial concentration of oxalacetate constant at 6.7×10^{-5} M. The inset in the lower left corner is a plot of the slopes of the three curves against the amount of tissue used.

FIG. 6. Log of $(H/v)-1$ plotted against the time of reaction with homogenate constant and oxalacetate variable; data from Fig. 1.

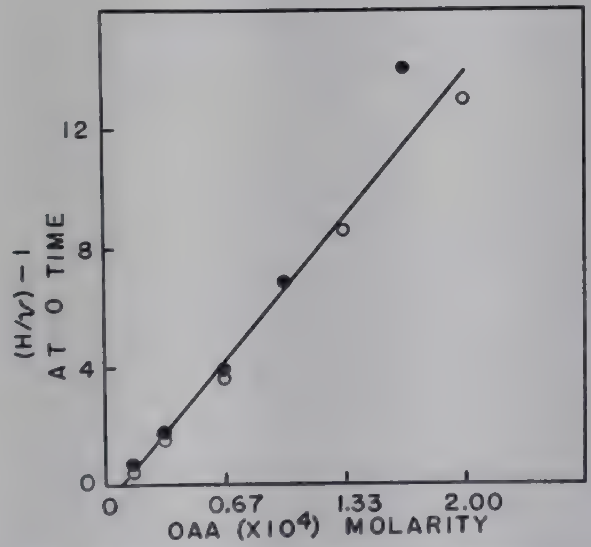


FIG. 7. Plot of values of $(H/v)-1$ at zero time against concentration of oxalacetate added. ○, data from Fig. 6; ●, from a similar experiment.

has been obtained (*cf.* Lichstein and Umbreit (13) p. 335, and Lardy *et al.* (14)). The experimental conditions described above may facilitate the attempts to study this problem.

The results with oxalacetate are of considerable interest in connection with an attractive suggestion by Pauling ((15) p. 58) that the surface configuration of the enzyme is complementary to the activated substrate rather than to the substrate itself. He proposed to test this postulate by searching for inhibitors that have a greater affinity for the enzyme than have the substrate molecules themselves. The succinic dehydrogenase system is one of the few that presents this opportunity; both malonate and oxalacetate have greater affinities than succinate for the enzyme. If ATP could convert oxalacetate to phosphoenol-oxalacetate, the configuration would be altered considerably, and no doubt to an extent that would prevent it from combining with the points having succinate affinity. This would seem to be the simplest explanation for the data at hand, but further experiments will have to be devised before it can be accepted.

SUMMARY

1. Oxalacetate inhibits the enzymatic oxidation of succinate. The dissociation constant of oxalacetate and enzyme is 1.5×10^{-6} M.

2. The oxalacetate gradually breaks down to pyruvate and the inhibition disappears.

3. Pyruvate decreases the rate of breakdown of oxalacetate, presumably by inhibiting the oxalacetic decarboxylase.

4. A mechanism is given based on competitive inhibition, and the first order rate of decarboxylation of oxalacetate.

5. ATP prevents the inhibition of the succinoxidase by oxalacetate, while the addition of calcium ions to an ATP-protected system restores the inhibition.

BIBLIOGRAPHY

1. Das, N. B., *Biochem. J.*, **31**, 1124 (1937).
2. Potter, V. R., *Ark. Kemi, Mineral. o. Geol.*, **13 B**, No. 7 (1939).
3. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **129**, 277 (1940).
4. Mann, P. J. G., and Quastel, J. H., *Biochem. J.*, **35**, 502 (1941).
5. Swingle, K. F., Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **145**, 581 (1942).
6. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, **149**, 217 (1943).
7. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943).
8. Potter, V. R., Pardee, A. B., and Lyle, G. G., *J. Biol. Chem.*, **176**, 1075 (1948).
9. Potter, V. R., and DuBois, K. P., *J. Gen. Physiol.*, **26**, 391 (1943).
10. Michaelis, L., and Menten, M. L., *Biochem. Z.*, **49**, 333 (1913).
11. Krebs, H. A., *Biochem. J.*, **36**, 303 (1942).
12. Rosenthal, O., *Biochem. J.*, **31**, 1710 (1937).
13. Lichstein, H. C., and Umbreit, W. W., *J. Biol. Chem.*, **170**, 329 (1947).
14. Lardy, H. A., Boyer, P. O., and Phillips, P. H., *Arch. Biochem.*, **5**, 295 (1944).
15. Pauling, L., *Am. Scientist*, **36**, 51 (1948).

XANTHINE OXIDASE AND TYROSINASE IN THE LIVERS OF CHICKS RECEIVING GRADED LEVELS OF DIETARY PTEROYLGLUTAMIC ACID*

BY CECILIA K. KEITH, WILSON J. BROACH, DORIS WARREN, PAUL L. DAY, AND JOHN R. TOTTER

(From the Department of Biochemistry, School of Medicine, University of Arkansas, Little Rock)

(Received for publication, July 26, 1948)

Evidence indicating a relationship between pteroylglutamic acid (PGA) and porphyrin metabolism has been presented previously from this laboratory (1). While this supposed relationship was being investigated, it was noted that in *in vitro* experiments conducted by the procedures of Schreus and Carrié (2) liver homogenate from PGA-deficient rats rapidly converted hemoglobin to methemoglobin, and that this conversion was inhibited by PGA. Upon subsequent incubation the PGA-containing mixtures produced consistently a somewhat smaller amount of porphyrin than did the controls not containing PGA.

Rodney, Swendseid, and Swanson (3) have reported that liver homogenate from PGA-deficient rats fed sulfonamides does not oxidize tyrosine at a normal rate. They also reported that added PGA increased the rate of oxidation under the conditions of their experiments. In somewhat similar experiments, with liver from PGA-deficient chicks, we have been unable to show any enhancement of tyrosine oxidation; instead, when PGA was added, we found a slight but consistent inhibition of oxygen uptake as compared with controls either in the presence or absence of tyrosine.

The recent publication by Kalckar of experiments indicating an inhibitory effect on xanthine oxidase by PGA (4) or by one of its breakdown products (5) appeared to offer an explanation for both of our observations discussed above. It is well known that methemoglobin may be formed during the oxidation of substrates of xanthine oxidase if hemoglobin is present. Presumably there would be enough of the precursors of endogenous uric acid present in the chick liver experiments to account for a small uptake of oxygen which might be inhibited by PGA. However, it has been found in later experiments[1] that xanthine oxidase may be only one of several flavin-containing enzymes which are inhibited by PGA or by its metabolic products. The later observation of Kalckar (5) that specially purified PGA exhibits but little inhibitory effect on xanthine oxidase makes it of importance to determine whether the *in vivo* action of the vitamin is related at all to this enzyme.

---

* Research paper No. 877, Journal Series, University of Arkansas. This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service, and a grant from the Williams-Waterman Fund of the Research Corporation.

[1] Totter, J. R., Martindale, W. E., and Keith, C. K., unpublished experiments.

The experiments described in this publication were designed to determine whether the liver xanthine oxidase of chicks is affected by dietary PGA. There are also presented here data on the oxygen uptake of liver homogenate from PGA-deficient chicks as affected by tyrosine and added PGA.

EXPERIMENTAL

White Leghorn chicks were obtained from a commercial hatchery and were placed on the experimental diets at approximately 2 days of age. They were housed in metal battery brooders and were given the experimental diets and water *ad libitum*. The diet was similar to that recently recommended for use in chick assays for the vitamin M group (6). It consisted of vitamin-free casein (Labco) 25 gm., gelatin 10 gm., corn-starch 52.4 gm., cellulose 3 gm., hydrogenated vegetable oil 3 gm., cod liver oil 1.3 gm., salts 5 gm. (7), L-cystine 0.3 gm., $MnSO_4 \cdot 4H_2O$ 0.1 gm., choline chloride 0.2 gm., thiamine chloride 0.4 mg., riboflavin 0.8 mg., pyridoxine hydrochloride 0.6 mg., 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine lactone[2] 0.05 mg., calcium pantothenate 1.1 mg., nicotinic acid 2.0 mg., *i*-inositol 5.0 mg., *p*-aminobenzoic acid 15.0 mg., 2-methylnaphthoquinone 0.005 mg., α-tocopherol 24.0 mg., and biotin[2] 0.01 mg.

All chicks were weighed weekly for 4 weeks. In the first series, groups of chicks received synthetic PGA[3] incorporated in the diet to the extent of 0, 5, 10, and 200 γ per 100 gm. of diet. Livers from these chicks were used for the tyrosinase determinations. Except for hematocrit determinations, no extensive hematological observations were made on the groups in this first series.

Xanthine oxidase was determined on the livers from a second series of chicks receiving 0, 5, 10, 20, 40, 80, 200, and 1000 γ of PGA per 100 gm. of diet. This series was also controlled by including a group which received a commercial chick starter diet[4] containing 1.75 γ of PGA per gm. by *Streptococcus faecalis* assay. In this second series, hemoglobin, hematocrit, total blood cell, and differential counts were made on each chick at the end of 4 weeks on the experiment. The chicks were then sacrificed and the organs removed for weighing. The livers were suspended in 5 volumes of phosphate buffer of pH 7.4 and homogenized in a Waring blendor.

---

[2] Kindly supplied by Merck and Company, Inc., Rahway, New Jersey.

[3] Kindly supplied by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

[4] Purina Startena.

The oxygen uptake of 1.0 ml. of 1:8 liver homogenate was measured at pH 7.5 in the absence of tyrosine and in the presence of 0.5 mg. of tyrosine, with the Warburg instrument. The total volume was 3.0 ml.

For the xanthine oxidase experiments 5 ml. of the liver mince from each chick were diluted to 25 ml. with phosphate buffer of pH 7.4. 2 ml. of the resulting mixture and 3 ml. of buffer were incubated with 5 ml. of a solution containing 5 mg. of xanthine in 0.01 N sodium hydroxide. A control with buffer instead of the xanthine solution was also set up. 1 ml. aliquots of these solutions taken immediately after mixing, and again after standing 1 hour at 32°, were treated with 1 ml. of 10 per cent sodium tungstate, 1 ml. of $\frac{2}{3}$ N sulfuric acid, and 7 ml. of water, and filtered. Uric acid was determined on 2 ml. aliquots of the filtrates by Brown's procedure (8). The xanthine oxidase activity was expressed as mg. of uric acid produced per hour per gm. of wet weight of liver.

RESULTS AND DISCUSSION

The average weights for the chicks in the second series are presented in Fig. 1. The hematological values were in close agreement with values obtained in an extensive series of experiments by Campbell and coworkers (9). The average weights and the hematocrit values obtained in our experiments, conducted at different seasons, were also in satisfactory agreement. More detailed observations on the blood and bone marrow of the chicks on these experiments will appear elsewhere.

Attention should be called to the ratio of the liver weight to body weights presented in Fig. 2. A large increase in the ratio occurs with diminishing intake of PGA. Similarly the deficient chicks had an obviously enlarged gallbladder as compared with positive controls. The gallbladder weights of eight negative control chicks averaged 0.77 gm., while those of eight chicks in the 40 γ of PGA group averaged 0.25 gm. The chicks on the commercial diet had a liver weight-body weight ratio of 0.0254, much lower than those receiving PGA far in excess of minimum requirements.

A typical tyrosinase experiment is given in Fig. 3. It may be seen that there is a decrease in oxygen consumption of the livers from deficient chicks in the presence of added PGA. This decrease is small; however, it was repeatedly observed in determinations made on livers of chicks receiving 0, 5, or 10 γ of PGA. No consistent effect of the dietary intake of PGA on the tyrosinase content of the livers was obtained. Also, there was no appreciable effect on tyrosinase found when PGA was added to the incubating mixtures as shown in Fig. 3. These results differ from those of Rodney, Swendseid, and Swanson (3) who made their observations on livers from sulfonamide-treated rats. The reasons for the differences in outcome between the two experiments are not apparent. Since there is obviously a relation between tyrosine metabolism and the action of PGA and of the antipernicious anemia principle (10, 11), caution in interpreting the results is indicated.

Results of the xanthine oxidase experiments are given in Fig. 4. An inverse relationship between PGA intake and xanthine oxidase is clearly shown. When the large liver weight-body weight ratio is taken into consideration, it may be seen that the deficient chicks possess an extremely high total oxidase activity.

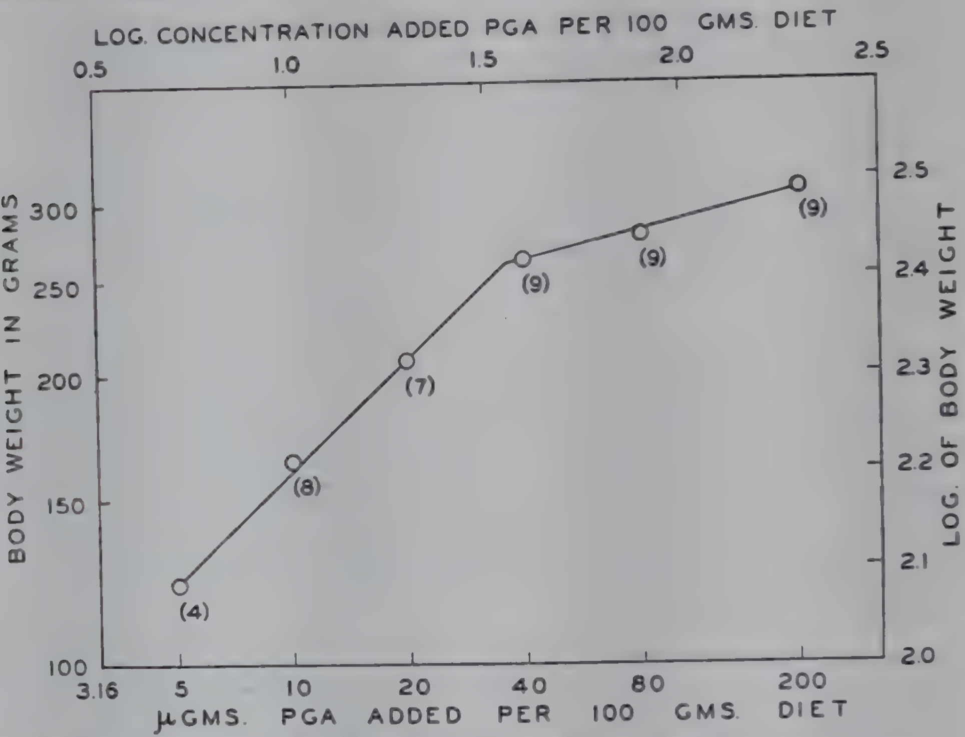


Fig. 1. The relation of the body weight of chicks to the pteroylglutamic acid content of the diet. The figures in parentheses are the number of chicks which survived the experimental period of 4 weeks. Initially the groups consisted of twenty, twelve, twelve, ten, ten, ten, nine, eight, and eight chicks for the 0, 5, 10, 20, 40, 80, 200, and 1000 γ, and commercial starter diet, respectively. The final average weight of the fourteen surviving negative control chicks was 114 gm., that for the eight receiving 1000 γ of PGA 247 gm., while the eight chicks on the commercial diet weighed an average of 286 gm. per chick.

Tests of the PGA used in our experimental diets indicate that it contains the impurity (5) which is responsible for the *in vitro* inhibition of xanthine oxidase. It may be argued that the impurity is responsible for the depression of the enzyme activity in the chick experiments. If so, the substance is of nutritional significance itself, and may occur in natural diets, since the xanthine oxidase activity of the livers from chicks on the commercial starter diet (0.94 mg. of uric acid per gm. per hour) was much lower than the lowest values obtainable with the supplemented purified diet. It is possible that additional dietary factors are present in the commercial diet which are necessary to maintain the low level of liver xanthine oxidase exhibited by the

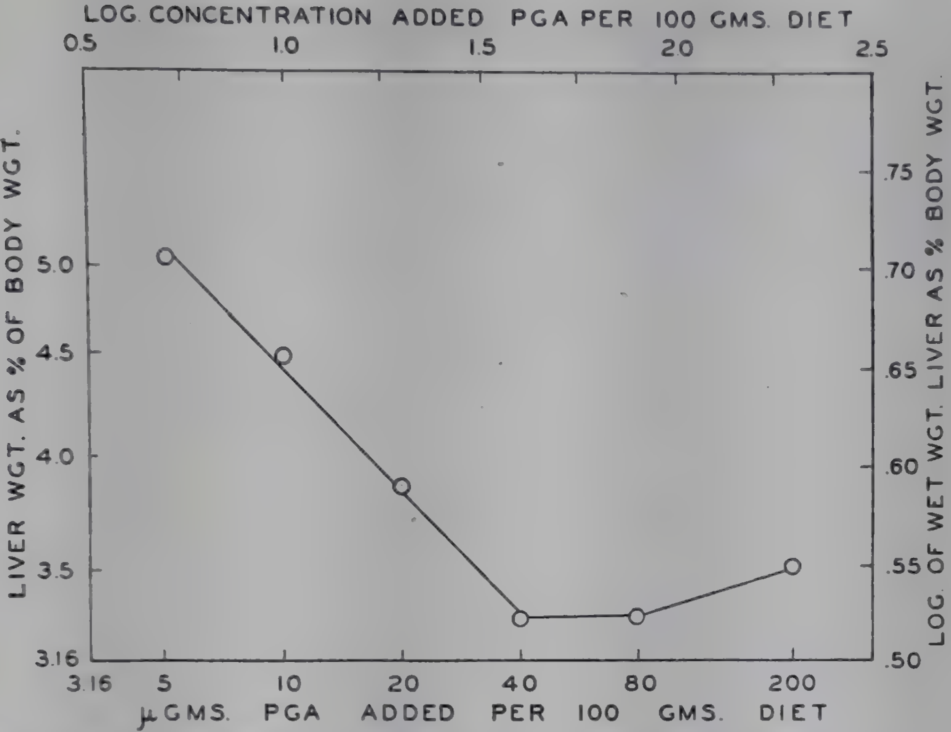


FIG. 2. The relative liver weight of chicks as affected by the PGA content of the diet. The numbers of chicks are the same as in Fig. 1. The fourteen negative control chick liver weights averaged 5.40 per cent, the eight of the 1000 γ group 2.96 per cent, and the eight of the commercial diet group 2.54 per cent of their body weights.

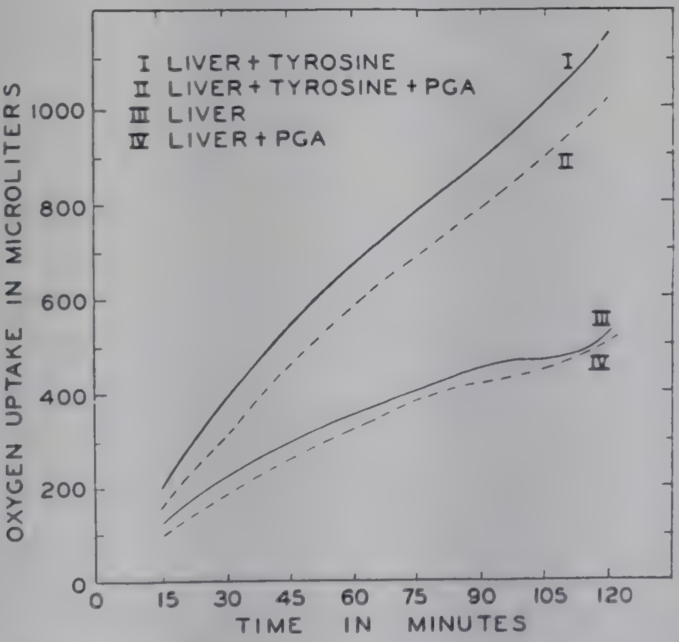


FIG. 3. The oxygen uptake of liver homogenate from PGA-deficient chicks in the presence and absence of added tyrosine. The abscissa gives the oxygen uptake in microliters per gm. of wet weight of liver. The liver used was a composite sample from three chicks.

chicks on such a diet. This phase of the problem is being investigated further.

The contribution which an excessively rapid rate of purine oxidation may make to the breakdown of hemoglobin or hemin-containing enzymes *in vivo* is unknown but may be of importance. The breakdown of hemoglobin is said to be brought about by hydrogen peroxide (12) and this compound is produced by the action of xanthine oxidase.

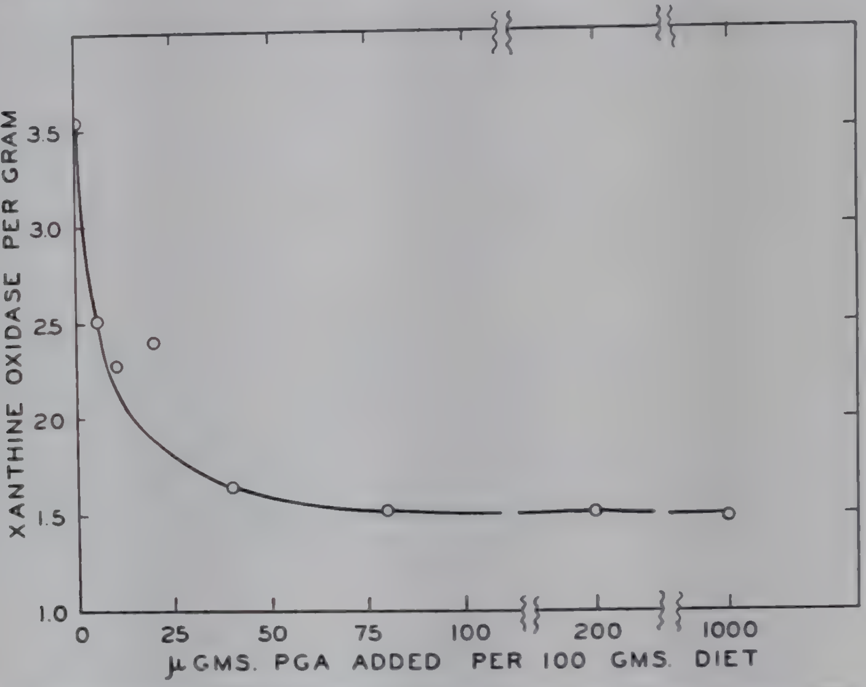


FIG. 4. The influence of PGA intake on chick liver xanthine oxidase. The abscissa gives the relative xanthine oxidase calculated as the mg. of uric acid produced from xanthine per gm. of wet weight of liver per hour. The number of determinations averaged for each point is the same as the number of chicks given in Fig. 1. The average xanthine oxidase for the fourteen negative control chicks was 3.55, while the eight on the commercial diet averaged 0.94.

It appears likely that other flavin-containing enzymes which catalyze the production of hydrogen peroxide as an end-product may also be involved in PGA deficiency and in the syndrome of pernicious anemia. Daft (13) and others (14) have presented results which may be interpreted as suggestive that L-amino acid oxidase activity is depressed by PGA. The recent findings of Woodruff and Darby (11) are possibly also explainable by such a hypothesis. An excessive rate of oxidative deamination of amino acids and the concurrent accelerated production of hydrogen peroxide could explain many of the biochemical changes in pernicious anemia and in PGA deficiency in the chick and in the monkey. The high liver weights and enlarged gallbladder of the deficient chicks suggest that an abnormally high rate of destruction of blood elements, or at least of materials normally used in blood production, does occur in PGA deficiency.

An important function of PGA and of liver extract may therefore be the regulation of the activity of flavin-containing enzymes such as xanthine oxidase, L-amino acid oxidase, D-amino acid oxidase, diaphorase, cytochrome reductase, etc. Evidence indicating a depressing effect of PGA on glycine oxidase will be presented shortly. Differences in the action of PGA and of liver extract may result from qualitative and quantitative differences in the ability of each to control adequately all of the enzymes involved.

SUMMARY

Weight data are presented on chicks receiving 0, 5, 10, 20, 40, 80, 200, or 1000 γ of pteroylglutamic acid (PGA) per 100 gm. of purified diet for a 4 week period.

The ratios of the chick liver weights to body weights were found to be inversely related to the PGA content of the purified diets. The gallbladders of the deficient chicks were greatly enlarged.

The oxygen uptake of liver homogenate from chicks receiving 0, 5, and 10 γ of pteroylglutamic acid per 100 gm. of diet was slightly depressed by addition of pteroylglutamic acid. No effect was observed on chick liver tyrosinase activity either by dietary PGA or by PGA added *in vitro*.

The chick liver xanthine oxidase was found to be inversely related to the PGA content of the purified diets. Excess of the vitamin did not reduce the average xanthine oxidase activity to a value as low as that exhibited by chicks on a commercial diet.

The bearing which the findings have on the interpretations of the biochemical changes occurring in pernicious anemia are briefly discussed.

BIBLIOGRAPHY

1. Steinkamp, R. C., Shukers, C. F., Totter, J. R., and Day, P. L., *Federation Proc.*, **6**, 295 (1947).
2. Schreus, H. T., and Carrié, C., *Klin. Wochschr.*, **13**, 1670 (1934).
3. Rodney, G., Swendseid, M. E., and Swanson, A. L., *J. Biol. Chem.*, **168**, 395 (1947).
4. Kalckar, H. M., and Klenow, H., *J. Biol. Chem.*, **172**, 349 (1948).
5. Kalckar, H. M., Kjeldgaard, N. O., and Klenow, H., *J. Biol. Chem.*, **174**, 771 (1948).
6. Day, P. L., and Totter, J. R., in Biological symposia, Lancaster, **12**, 313 (1947).
7. Jones, J. H., and Foster, C., *J. Nutr.*, **24**, 245 (1942).
8. Brown, H., *J. Biol. Chem.*, **158**, 601 (1945).
9. Campbell, C. J., McCabe, M. M., Brown, R. A., and Emmett, A. D., *Am. J. Physiol.*, **144**, 348 (1945).
10. Swendseid, M. E., Burton, I. F., and Bethell, F. H., *Proc. Soc. Exp. Biol. and Med.*, **52**, 202 (1943).
11. Woodruff, C. W., and Darby, W. J., *J. Biol. Chem.*, **172**, 851 (1948).
12. Granick, S., and Gilder, H., in Nord, F. F., Advances in enzymology and related subjects, **7**, 305 (1947).
13. Daft, F. S., *Federation Proc.*, **6**, 405 (1947).
14. Shehata, O., and Johnson, B. C., *Proc. Soc. Exp. Biol. and Med.*, **67**, 332 (1948).

CALCIFICATION OF TEETH

I. COMPOSITION IN RELATION TO BLOOD AND DIET*

By ALBERT E. SOBEL and ALBERT HANOK

(*From the Department of Biochemistry and the Pediatric Research Laboratory, The Jewish Hospital of Brooklyn, New York*)

(Received for publication, July 27, 1948)

These investigations were undertaken to test the *hypothesis*, of the authors, that there is a *relationship between the inorganic composition of teeth and the fluid from which the tooth salts precipitate and that the composition of this fluid in turn is related to the blood serum.* If this hypothesis is valid, it should be possible to vary the composition of teeth (during the period of active lime salt deposition), since the composition of blood serum can be regulated by the diet (1–6).

The mineral phase is regarded as essentially the same in teeth and bone (7, 8). The variations in the composition of bone are well known (9) and were shown to be related to that of blood (5, 6). Hitherto no such relationships have been shown for teeth. In fact, some recent investigations indicate that the inorganic composition of enamel is constant (10). There is, however, evidence that teeth are tissues in equilibrium with body fluids. Barnum and Armstrong (11) fed P^{32} to cats and found that it was present in both enamel and dentin after an interval. They then postulated that dentin phosphate may be in equilibrium with blood phosphate and that enamel phosphate may be in equilibrium with dentin phosphate. Greenberg (12) administered Ca^{45} to rachitic rats and found that in the absence of vitamin D the uptake of the isotope in the femurs was greater than in the molars but less than in the incisors. In the presence of vitamin D, the uptake was greatest in the femur, less in the incisors, and least in the molars. Vitamin D had a minor influence on teeth but a major influence on bone. Armstrong and Barnum (13) fed Ca^{45} and P^{32} to albino rats and measured the amounts found in the teeth. They found that the turnover of the isotopes in the incisor dentin was two-thirds and in the incisor enamel about one-third of that in the femur. The molar dentin was about one-sixth that of the femur and the molar enamel was from 1.5 to 3 per cent of the femur.

These studies with isotopes indicate that the continually growing incisors may undergo changes almost as rapidly as bone and thus were considered suitable for our experiments. It is worth pointing out that the hypothesis proposed in the first paragraph applies to teeth only during the period when active calcification takes place. In human teeth active calcification is almost complete before eruption.

---

* Presented in part before the Division of Biological Chemistry at the 112th meeting of the American Chemical Society, New York, September 15–19, 1947.

The knowledge of the factors that influence the composition of teeth is of theoretical and practical importance, since further studies may reveal some relation between caries and structural, mechanical, and other properties (such as resistance to acids and bacteria) and composition.

In the experiments reported here, evidence is given that there is a relationship between the $PO_4:CO_3$ ratios of enamel, dentin, and blood serum, which in turn is regulated by the diet.

EXPERIMENTAL

The three diets used are shown in Table I. The basal diet contained traces of calcium (0.029 per cent) and suboptimal amounts of phosphorus (0.118 per cent).[1] To this basal diet 3.0 per cent $CaCO_3$ was added to produce a high calcium-low phosphorus diet (Diet B); 3.0 per cent of anhydrous Na_2HPO_4 was added to give a low calcium-high phosphorus diet (Diet C); and 0.4 per cent $CaCO_3$ was added to give the low calcium-low phosphorus diet (Diet D). The calcium and phosphorus contents of the diets were determined by replicate analysis. Each diet contained the same amount of either calcium or phosphorus as one of the others.

Young rats, 23 days old, were used. The animals were raised in our laboratory from a Wistar strain stock colony, fed on the standard stock diet of Bills *et al.* (14). Litters were chosen having at least eight animals per litter. Two animals of each litter were sacrificed on the 1st day of the experiment to furnish data on the initial composition of the blood and teeth of the litter. These animals were termed the reference group. It was necessary, owing to the small size of the young animals, to pool the blood and teeth of both animals for analysis. The remaining six animals in each litter were divided into three pairs and each pair placed on one of the three experimental diets. In addition to these diets, one animal of each pair received daily 100 I.U. of vitamin D in 0.1 ml. of corn oil administered orally by means of a pipette. The six groups thus established were caged separately, in a darkened room, to prevent mixing of the diets and to prevent the access of those animals not receiving vitamin D to this factor.

Eleven litters of rats were used. The mean initial weights of the rats were almost identical (see Table II). A reasonable basis for comparing the results of the various groups was established because of the similarity of heredity, the distribution of litters among the various groups, and the close agreement of the initial weights.

---

[1] In these experiments a basal diet, low in phosphate, was desired to obtain greater freedom in changing the Ca:P ratio. This was accomplished by selecting degerminated yellow corn-meal which is low in phosphorus (Hoger Cereal Company, New York).

The first group consisting of six litters, and referred to as the short term experiment, was kept on the experimental diets for 30 days, at the end of which time the rats were sacrificed by cutting the jugular vein and the carotid artery. The blood was collected under oil and centrifuged immediately upon clotting. The upper incisors were immediately removed, cleaned from adhering tissues, and prepared for analysis. The second group of animals, consisting of five litters (referred to as the long term experiment), was treated exactly the same as the animals on the short term experiment except that they were kept on the experimental diets for 45 days.

TABLE I

Composition of Experimental Diets

Diet	Constituents		Ca	P	Ca:P
		parts	*per cent*	*per cent*	*mole ratio*
B. High calcium-low phosphorus	Basal diet	97			
	Calcium carbonate	3	1.20	0.121	7.67
C. Low calcium-high phosphorus	Basal diet	97			
	Na_2HPO_4	3	0.028	0.759	0.029
D. Low calcium-low phosphorus	Basal diet	99.6			
	Calcium carbonate	0.4	0.189	0.124	1.18
Basal	Yellow corn-meal	75			
	Wheat gluten	19			
	Brewers' yeast	5			
	Sodium chloride	1	0.029	0.118	0.191

Analytical Procedures

The serum, under oil, was analyzed for CO_2 on 0.1 ml. by the manometric method of Van Slyke and Neill (15), for calcium in duplicate on 0.1 ml. portions by the method of Sobel and Sobel (16), and for inorganic phosphate in duplicate on 0.1 ml. portions by the micro modification of the Fiske-Subbarow method (17, 18), with the micro cups of the Klett-Summerson photoelectric colorimeter with a 660 mμ filter. Standards were run for CO_2, calcium, and inorganic phosphate for each set of sera. Standards for CO_2 consisted of a sodium carbonate solution, 1 ml. of which is equivalent to 100 volumes per cent of CO_2. The standard for calcium consisted of a calcium carbonate solution, 0.1 ml. of which is equivalent to 10.00 γ of calcium. The standard for inorganic phosphate consisted of a solution of KH_2PO_4, 5 ml. of which are equivalent to 40.0 γ of P. The precision of the methods is shown in Table III.

The upper incisors were split into fragments and extracted for three overnight periods with two portions of acid- and aldehyde-free ethyl alcohol and one portion of ethyl ether. The fragments were then dried overnight at 105°, weighed, and the enamel and dentin separated by the method of Manly and Hodge (19), which was modified to the extent that the tube used for carbonate analysis (20) was employed instead of the centrifuge tube recommended by Manly and Hodge. This was done to obviate the neces-

TABLE II

Growth data

	Dietary group	Initial weight	Final weight	Gain
		gm.	gm.	gm.
Experimental period, 30 days	Reference group	+30.1		
	B	+31.1	+55.0	+23.9
	C	+31.2	+44.5	+13.3
	D	+30.0	+64.9	+34.9
	B + vitamin D	+30.6	+43.6	+13.0
	C + “ “	+30.3	+48.5	+18.2
	D + “ “	+29.7	+73.5	+43.8
	B – C	–0.1	+11.5	+10.6
	“ – D	+1.1	–9.9	–11.0
	C – “	+1.2	–20.4	–21.6
	B + vitamin D – C + vitamin D	+0.3	–4.9	–5.2
	“ “ “ – D + “ “	+0.9	–29.9	–30.8
	C + “ “ – “ + “ “	+0.6	–25.0	–25.6
	B – (B + vitamin D)	+0.5	+11.4	+10.9
	C – (C + “ “)	+0.9	–4.0	–4.9
	D – (D + “ “)	+0.3	–8.6	–8.9
Experimental period, 45 days	Reference group	+35.6		
	B	+36.8	+51.9	+15.1
	C	+36.6	+45.9	+9.3
	D	+35.8	+82.1	+46.3
	B + vitamin D	+36.1	+43.4	+7.3
	C + “ “	+36.1	+64.5	+28.4
	D + “ “	+36.8	+88.4	+51.6
	B – C	+0.2	+6.0	+5.8
	“ – D	+1.0	–30.2	–31.2
	C – “	+0.8	–36.2	–37.0
	B + vitamin D – C + vitamin D	0.0	–21.1	–21.1
	“ + “ “ – D + “ “	–0.7	–45.0	–44.3
	C – “ “ – “ + “ “	–0.7	–23.9	–23.2
	B – (B + vitamin D)	+0.7	+8.5	+7.8
	C – (C + “ “)	+0.5	–18.6	–19.1
	D – (D + “ “)	–1.0	–6.3	–5.3

sity of transferring small amounts of enamel and dentin obtained from the teeth, with the consequent mechanical loss of material. The separated enamel and dentin were dried overnight at 105°, after two acetone washings, and placed in a desiccator till cool and then weighed. These weights were used to calculate the percentages of the inorganic constituents of the enamel and dentin.

The enamel was analyzed for carbonate, calcium, phosphate, and total base as previously described (18), except that the Coleman spectrophotometer was used instead of the visual colorimeter for phosphate. For the carbonate analysis, the gasometric reagents were prepared in 25 per cent sodium chloride for added sensitivity (21). Standard solutions were run for calcium, phosphate, total base, and for carbonate solid mixtures. The latter consisted of a dry sodium chloride-sodium carbonate mixture containing 2.00 per cent CO_3. The precision of these determinations is given in Table III.

TABLE III

Precision of the Determinations of Standard Solutions Employed As Controls for Enamel, Dentin, and Blood Serum Analysis

Mean values (approximately 50 determinations plus-minus average deviations).

Ca*	Ca†	CO_2‡	CO_3§	PO_4‖	Total base¶
γ	γ	*vol. per cent*	*wt. per cent*	γ	*m.eq.*
10.03 ± 0.12	199.0 ± 1.7	100.0 ± 1.3	2.00 ± 0.02	39.0 ± 0.5	0.101 ± 0.001

* 10.00 γ of Ca present in 0.1 ml. used for analysis.

† 200 γ of Ca present in 1.0 ml. of solution used for analysis (bone method).

‡ 100.0 volumes per cent of CO_2 present; 0.1 ml. used for analysis.

§ 2.00 per cent as CO_3 in a mixture of solid NaCl and Na_2CO_3 (bone method).

‖ 40.0 γ of phosphate P present (bone and blood methods).

¶ 0.100 milliequivalent of Ca present in 1.00 ml.

Calculations (6, 20)

Carbonate

$$\text{Mg. } CO_3 \text{ in sample} = P_{CO_2} \times \text{factor for } CO_3$$

$$P_{CO_2} = p_1 - p_2 - c$$

$$c = p_1 - p_2 \text{ for blank analysis}$$

Calcium

$$\text{Mg. Ca in sample} = \frac{\text{titer} \times \text{normality of acid} \times 20 \times \text{total volume}}{\text{volume of aliquot used}}$$

Phosphate

Klett-Summerson colorimeter:

$$\text{Mg. P in aliquot} = \frac{\text{reading of unknown}}{\text{reading of standard}} \times \text{concentration of standard}$$

For the Coleman spectrophotometer (used for tooth analysis) a calibration curve was employed.

$$\text{Mg. P in sample} = \text{mg. P in aliquot} \times \frac{\text{total volume of solution}}{\text{volume of aliquot used}}$$

Total Base

$$\text{Milliequivalents total base} = \frac{\text{titer} \times \text{normality of acid} \times \text{total volume of solution}}{\text{volume of aliquot used}}$$

mM Ca = (mg. Ca in enamel or dentin)/40.08
" PO_4 = (mg. inorganic P in enamel or dentin)/31.02
" CO_3 = (mg. CO_3 in enamel or dentin)/60.01
" % Ca, PO_4, CO_3, or total base = (mM Ca, PO_4, CO_3, or total base × 100)/mg. weight of dry, fat-free enamel or dentin
Ca:PO_4 ratio = mM Ca:mM PO_4
mM residual Ca = mM total Ca minus mM CO_3
Milliequivalents residual $\frac{\text{total base}}{2}$ = milliequivalents $\frac{\text{total base minus mM } CO_3}{2}$
Residual Ca:PO_4 = mM residual Ca:mM PO_4
" total base: $2PO_4$ = milliequivalents residual total base: 2 mM PO_4
PO_4:$2CO_3$ = mM PO_4:2 mM CO_3

The $PO_4:2CO_3 = n$ in the usual way of representing the inorganic composition of bone and teeth $[Ca_3(PO_4)_2]_n[CaCO_3]_{1.0}$ (7, 8) when the molar ratio of residual Ca:PO_4 is 1.50. This holds true also when the residual Ca:PO_4 molar ratio is more than 1.50 if we take the $CaCO_3$ as unity. The formula in this case would be $[Ca_3(PO_4)_2]_n[CaCO_3]_{1.0}[CaX]_y$. When the residual Ca:$PO_4$ molar ratio is less than 1.50, we have the type of formula represented by $[Ca_3(PO_4)_2]_n[CaCO_3]_{1.0}[CaHPO_4]_x$ and n is then overstated.

The mean results of the enamel and dentin analysis were evaluated by the statistical methods of Fisher (22) as applied to small samples. P represents the probability that the difference between two means is due to chance. When P has a value less than 0.05, the difference between two means is statistically significant.

Weight Changes

The weight changes, including the initial and final weights are given in Table II. Both in the absence and in the presence of vitamin D, Diet D gave the best growth. This is in harmony with the fact that dietary calcium to phosphorus ratios between 1.0 and 2.0 are optimum for best growth (1–3). The absence of vitamin D gave better growth in Group C than in Group D, and in the presence of vitamin D, the reverse was the case. In previous experiments (5) better growth was obtained in Group B than in Group C, since the phosphate level was 0.32 per cent instead of the present 0.12 per cent.

The influence of vitamin D was greatest in Group C. In Group B, there was a reduction which again may be explained on the basis of the relatively high calcium-low phosphorus ratio. On such diets vitamin D diverts the available phosphorus from the soft tissues to the bone. In Group D, in which growth was greatest, the addition of vitamin D had no significant effect. In the absence of vitamin D the rickets produced in Group B were severe and in Group C mild.

TABLE IV

Composition of Serum in Relation to Diet (Mean Values)

	Dietary group	Ca	P	CO_2	Ca × P	$Ca:PO_4$	$PO_4:CO_3$	Dietary Ca:P
		mg. per cent	*mg. per cent*	*vol. per cent*		*molar ratio*	*molar ratio*	*molar ratio*
Experimental period, 30 days	B	11.7	2.1	60.6	23.1	4.32	0.025	7.67
	C	5.6	7.5	53.7	41.8	0.58	0.101	0.029
	D	9.4	4.7	58.0	44.1	1.55	0.057	1.18
	B + vitamin D	13.3	3.4	62.6	44.9	3.03	0.039	7.67
	C + " "	8.8	8.4	57.6	74.2	0.81	0.108	0.029
	D + " "	11.1	6.0	56.0	65.8	1.43	0.076	1.18
	Reference	11.2	9.5	47.6	105.8	0.92	0.148	
Experimental period, 45 days	B	12.3	1.8	54.1	22.2	5.30	0.024	7.67
	C	5.1	4.9	57.7	25.0	0.81	0.062	0.029
	D	10.0	2.6	50.6	26.0	2.98	0.037	1.18
	B + vitamin D	15.0	3.4	57.6	51.0	3.42	0.043	7.67
	C + " "	8.9	5.2	56.2	46.4	1.33	0.067	0.029
	D + " "	11.2	4.6	55.6	51.5	1.89	0.060	1.18
	Reference	10.9	7.7	53.9	83.8	1.10	0.104	

Composition of Blood, Enamel, and Dentin

Blood Serum and Diet—The results obtained from the analysis of the blood sera are presented in Table IV. There is a direct relationship in the order of magnitude of serum $Ca:PO_4$ and dietary Ca:P ratios. There is an inverse relationship in the order of magnitude of serum $PO_4:CO_3$ and dietary Ca:P ratios. Vitamin D raised the Ca × P product in all cases, the per cent rise being more in that member of the calcium-phosphorus pair which was low compared to the normal, but did not completely overcome the influence of the diet. For this reason the differences between the maxima and minima in the $Ca:PO_4$ and the $PO_4:CO_3$ ratios are less, though still in the same order as in the absence of vitamin D. These relationships confirm, even more definitely, the results of previous investigators (1–6).

Deposition of Inorganic Components As Indicated by Percentages Present—It is evident from Table V that the density of calcium, carbonate, and total base is decisively higher in the enamel than in the dentin in all cases.

The density of phosphorus is higher in all but two cases. This confirms previous work indicating higher density in enamel (19, 23–26). Dietary calcium, phosphorus, and vitamin D were not decisive in influencing the density of either enamel or dentin, though in the long term experiment the carbonate was highest in Group B and lowest in Group C.

Enamel, Dentin, and Blood Serum—The composition of enamel and dentin in relation to blood serum and diet is given in Table VI and the statistical evaluation of the data in Table VII. One is impressed by the fact that the differences that existed in composition were magnified in the long term experiment. The $PO_4:CO_3$ ratios in dentin and enamel arrange themselves in the same order as the serum $PO_4:CO_3$ ratios for the three groups not receiving vitamin A. The same holds true for the three groups receiving vitamin D. When all six groups of the short term experiment are arranged in the order of magnitude of serum $PO_4:CO_3$ ratios, the $PO_4:CO_3$ ratios in both dentin and enamel follow the same order. In the long term experiment the dentin and enamel $PO_4:CO_3$ and serum $PO_4:CO_3$ ratios do not show the same pattern as in the short term experiment when all six groups are arranged in order of magnitude. This may be indirect evidence that vitamin D has an influence. Statistically, the most significant differences are between the two extreme groups; namely, Group B (high calcium-low phosphorus) and Group C (low calcium-high phosphorus).

TABLE V

Composition of Enamel and Dentin (Mean Values)

The results for total base are given in milliequivalents per cent × 100; for Ca, PO_4, and CO_3 in mM per cent × 100.

	Dietary group	Enamel				Dentin			
		Total base	Ca	PO_4	CO_3	Total base	Ca	PO_4	CO_3
Experimental period, 30 days	B	200.0	96.6	50.6	5.31	124.3	61.2	43.6	5.05
	C	199.5	94.3	55.6	5.20	111.3	54.1	39.3	3.67
	D	224.2	108.9	56.0	5.02	118.7	58.5	45.1	4.27
	B + vitamin D	149.0	71.5	40.8	6.57	116.8	57.3	41.5	4.56
	C + " "	166.3	80.3	46.4	4.37	117.5	58.2	44.9	3.83
	D + " "	186.0	87.3	51.7	5.64	119.2	59.0	44.1	4.12
	Reference	219.0	102.2	47.8	5.99	125.3	59.6	45.2	3.61
Experimental period, 45 days	B	181.4	90.1	45.2	6.17	133.8	64.2	44.1	4.15
	C	196.2	96.1	51.5	3.47	124.6	61.5	44.2	2.48
	D	188.6	93.5	46.4	4.00	143.0	68.1	50.4	3.63
	B + vitamin D	188.5	93.2	50.3	6.35	130.8	63.4	46.6	4.46
	C + " "	198.3	98.5	55.1	4.01	132.3	65.4	48.1	3.02
	D + " "	191.6	94.7	47.9	4.32	135.4	67.2	47.2	3.39
	Reference	169.4	84.5	49.8	7.78	124.6	59.9	44.3	3.62

The $PO_4:CO_3$ ratios of the enamel in all cases are lower than of the corresponding dentin. The difference was most marked in the high calcium-low phosphorus group (Diet B) and least in the low calcium-high phosphorus group (Diet C). In fact the differences in the absence of vitamin D for this group were not statistically significant, though all other differences were statistically significant for corresponding enamels and dentins. In this connection one can point out that the differences in composition between enamel and dentin of humans and dogs (26) and rats (27) may be due to the particular diet given rather than to species differentiation.

TABLE VI

Composition of Enamel and Dentin in Relation to Blood Serum and Diet (Mean Values)

The results represent molar ratios.

	Dietary group	Dietary $Ca:PO_4$	Serum		Enamel		Dentin	
			$Ca:PO_4$	$PO_4:CO_2$*	$Ca:PO_4$	$PO_4:2CO_3$	$Ca:PO_4$	$PO_4:2CO_3$
Experimental period, 30 days	B	7.67	4.32	2.51	1.92	2.08	1.41	4.40
	C	0.029	0.58	10.10	1.68	4.60	1.38	5.43
	D	1.18	1.55	5.86	1.95	2.65	1.31	5.15
	B + vitamin D	7.67	3.03	3.94	1.75	2.43	1.38	4.60
	C + " "	0.029	0.81	10.80	1.74	3.68	1.29	5.98
	D + " "	1.18	1.43	7.75	1.70	3.00	1.36	5.41
	Reference		0.92	14.80	2.20	3.25	1.31	6.56
Experimental period, 45 days	B	7.67	5.30	2.41	1.99	3.71	1.51	5.47
	C	0.029	0.81	6.15	1.85	7.72	1.41	9.31
	D	1.18	2.98	3.72	2.01	5.92	1.36	6.87
	B + vitamin D	7.67	3.42	4.27	1.86	3.62	1.39	5.55
	C + " "	0.029	1.33	6.70	1.77	6.94	1.36	7.97
	D + " "	1.18	1.89	5.99	1.98	5.67	1.41	7.27
	Reference		1.10	10.40	1.68	3.34	1.39	4.88

* × 100.

Not until we understand the influence of diet in a given species can we discuss the differences between enamel and dentin as characteristic of different species.

Though $Ca:PO_4$ ratios of serum undergo wide variations, in the enamel and dentin the ratios undergo no corresponding variations. Statistically there were significant differences in the two extreme groups; namely, the high calcium-low phosphorus and the low calcium-high phosphorus group (Diets B and C). In the short term experiment these differences were so only in the enamel, while in the long term experiment they were true for both enamel and dentin. In our earlier studies on bone (6), significant differences were obtained only between the two extreme diets (Diets A and B) in the absence of vitamin D.

The Ca:PO$_4$ ratio in the enamel is significantly higher, in all cases, than the ratio for the corresponding dentin. This would imply that if we assume the apatite formula, $[Ca_3(PO_4)_2]_n[CaX]$, X, in addition to carbonate, must have some other radical in the enamel, possibly citrate, which was reported (28), or fluoride, and in the dentin it must be some phosphate, probably $HPO_4^{=}$ (6, 29).

The ratios of residual total base to 2PO$_4$ and residual Ca:PO$_4$ are given

TABLE VII

Statistical Evaluation of Ratios Presented in Table VI (P Values)

All P values are less than the numbers shown.

Line No.	Dietary group	Serum		Enamel		Dentin		Enamel *vs.* dentin	
		Ca:PO$_4$	PO$_4$:CO$_3$	Ca:PO$_4$	PO$_4$:2CO$_3$	Ca:PO$_4$	PO$_4$:2CO$_3$	Ca:PO$_4$	PO$_4$:2CO$_3$
	Experimental period, 30 days								
1	B *vs.* C	0.01	0.01	0.02	0.02	0.4	0.02		
2	" " D	0.01	0.01	0.9	0.05	0.01	0.2		
3	C " "	0.01	0.01	0.2	0.05	0.02	0.6		
4	B + vitamin D *vs.* C + vitamin D	0.01	0.01	0.6	0.1	0.02	0.01		
5	B + vitamin D *vs.* D + vitamin D	0.01	0.01	0.7	0.5	0.6	0.02		
6	C + vitamin D *vs.* D + vitamin D	0.01	0.1	0.9	0.5	0.2	0.2		
7	B *vs.* B + vitamin D	0.05	0.05	0.2	0.2	0.2	0.5		
8	C " C + " "	0.01	0.6	0.7	0.5	0.05	0.2		
9	D " D + " "	0.6	0.1	0.1	0.7	0.3	0.6		
10	Reference *vs.* B	0.01	0.01	0.5	0.2	0.02	0.01		
11	" " C	0.1	0.01	0.2	0.3	0.1	0.2		
12	" " D	0.01	0.01	0.6	0.4	1.00	0.1		
13	" " B + vitamin D	0.01	0.01	0.2	0.3	0.05	0.01		
14	" " C + " "	0.2	0.01	0.3	0.7	0.7	0.5		
15	" " D + " "	0.01	0.01	0.2	0.9	0.4	0.2		
16	B							0.01	0.01
17	C							0.01	0.3
18	D							0.01	0.01
19	B + vitamin D							0.01	0.01
20	C + " "							0.01	0.01
21	D + " "							0.01	0.01
22	Reference							0.02	0.01

TABLE VII—*Concluded*

Line No.	Dietary group	Serum		Enamel		Dentin		Enamel *vs.* dentin	
		$Ca:PO_4$	$PO_4:CO_3$	$Ca:PO_4$	$PO_4:2CO_3$	$Ca:PO_4$	$PO_4:2CO_3$	$Ca:PO_4$	$PO_4:2CO_3$
	Experimental period, 45 days								
1′	B *vs.* C	0.01	0.01	0.01	0.01	0.02	0.01		
2′	" " D	0.05	0.01	0.9	0.01	0.05	0.02		
3′	C " "	0.01	0.01	0.05	0.05	0.8	0.02		
4′	B + vitamin D *vs.* C + vitamin D	0.05	0.01	0.9	0.01	0.6	0.01		
5′	B + vitamin D *vs.* D + vitamin D	0.1	0.3	0.2	0.01	0.8	0.1		
6′	C + vitamin D *vs.* D + vitamin D	0.01	0.6	0.01	0.05	0.4	0.3		
7′	B *vs.* B + vitamin D	0.2	0.2	0.2	0.8	0.1	0.9		
8′	C " C + " "	0.01	1.00	0.8	0.4	0.7	0.2		
9′	D " D + " "	0.1	0.1	0.7	0.6	0.5	0.1		
10′	Reference *vs.* B	0.01	0.01	0.02	0.2	0.02	0.3		
11′	" " C	0.01	0.01	0.2	0.01	0.4	0.01		
12′	" " D	0.01	0.01	0.01	0.01	0.6	0.01		
13′	" " B + vitamin D	0.01	0.01	0.1	0.3	1.0	0.01		
14′	" " C + " "	0.01	0.01	0.3	0.01	0.2	0.01		
15′	" " D + " "	0.01	0.01	0.01	0.01	0.8	0.01		
16′	B							0.01	0.01
17′	C							0.01	0.2
18′	D							0.01	0.05
19′	B + vitamin D							0.01	0.05
20′	C + " "							0.01	0.02
21′	D + " "							0.01	0.05
22′	Reference							0.01	0.01

in Table VIII. It is evident that the ratios are still above 1.50 for enamel and below 1.50 for dentin. Thus the differences cannot be accounted for on the basis of the high carbonate content of the enamel.

Another point worth mentioning is that the difference between total base and calcium is not as great as that in bone (6).

Some differences in the two reference groups, shown in Table VI, need to be pointed out. The $Ca:PO_4$ ratio of the enamel and of the dentin is much higher in the first reference group than in the second. The rats in the two reference groups were raised about 10 months apart and differences in them may be accounted for by the fact that the stock diet used (14) is made up of dried milk, crude casein, yellow corn, alfalfa, cottonseed meal, brewers' yeast, and salt. Each one of the components, except the salt, can undergo wide variations in composition from batch to batch and thus may cause changes in the fluids that feed the embryo (of which practically nothing is known at the present time) and in the composition of the mother's milk, which is known to undergo change (30).

Table VIII

Comparison of Residual Total Base to PO_4 and Residual Ca to PO_4 of Enamel and Dentin (Mean Values)

Dietary group	Enamel						Dentin					
	Residual $\frac{T.B.}{2}$	Residual Ca	T.B.:2PO_4	Ca:PO_4	Residual T.B.:2PO_4	Residual Ca:PO_4	Residual $\frac{T.B.}{2}$	Residual Ca	T.B.:2PO_4	Ca:PO_4	Residual T.B.:2PO_4	Residual Ca:PO_4
Experimental period, 30 days												
	m.eq. per cent × 100	*mM per cent × 100*	*molar ratio*	*molar ratio*	*molar ratio*	*molar ratio*	*m.eq. per cent × 100*	*mM per cent × 100*	*molar ratio*	*molar ratio*	*molar ratio*	*molar ratio*
B	95.7	91.3	1.97	1.92	1.89	1.80	57.1	56.1	1.43	1.41	1.31	1.29
C	94.6	89.1	1.79	1.68	1.70	1.60	52.0	50.5	1.42	1.38	1.32	1.28
D	107.1	103.9	2.00	1.95	1.92	1.86	55.1	54.2	1.32	1.31	1.22	1.20
B + vitamin D	67.9	64.9	1.82	1.75	1.66	1.59	53.8	52.7	1.41	1.38	1.30	1.27
C + " "	78.7	75.9	1.79	1.74	1.70	1.63	55.0	54.4	1.31	1.29	1.23	1.21
D + " "	87.4	81.7	1.82	1.70	1.69	1.58	55.5	54.9	1.35	1.34	1.26	1.25
Reference	103.5	96.2	2.29	2.20	2.17	2.01	59.1	56.0	1.39	1.31	1.31	1.24
Experimental period, 45 days												
B	84.5	83.9	2.00	1.99	1.87	1.86	62.7	60.0	1.52	1.51	1.42	1.36
C	94.6	92.6	1.90	1.85	1.84	1.80	59.8	59.0	1.41	1.41	1.35	1.33
D	90.0	89.5	2.03	2.01	1.94	1.93	67.9	64.5	1.42	1.36	1.35	1.28
B + vitamin D	87.9	86.6	1.87	1.86	1.74	1.72	60.9	58.9	1.40	1.39	1.31	1.26
C + " "	95.2	94.5	1.80	1.77	1.73	1.71	63.2	62.4	1.38	1.36	1.31	1.30
D + " "	91.5	90.4	2.00	1.98	1.91	1.89	64.3	63.8	1.44	1.41	1.36	1.35
Reference	76.9	76.7	1.70	1.68	1.54	1.54	58.7	56.6	1.40	1.39	1.33	1.28

T.B. = total base.

DISCUSSION

It is evident from these experiments that there is a relationship between the PO_4:CO_3 ratio of blood serum and that of enamel and dentin; this is illustrated in Table IX. For enamel and dentin the PO_4:CO_3 ratio in those groups without vitamin D follows that for serum. The same holds true for those groups given vitamin D. These three groups are arranged in a descending order of dietary Ca:P ratios. Thus the relationship of diet, blood, and teeth is evident. This relationship, in the 30 day experiment, between blood and teeth is complete. In the 45 day experiment, however, we must segregate the vitamin D-fed groups from the non-vitamin D groups. Vitamin D may manifest an influence that is evident only in the older animals. The effect of vitamin D is not as great on the tooth PO_4:CO_3 ratios as one might expect from the changes in the serum PO_4:CO_3 ratios. In this connection it is worth mentioning that the density of neither enamel nor dentin was influenced by vitamin D. The rate of turnover of isotopic calcium and phosphorus in the teeth is not markedly influenced by vitamin D (12, 13). This points to an important difference between tooth and bone; namely, that in bone vitamin D exerts a marked influence on the PO_4:CO_3 ratio, density of calcification, and rate of turnover of calcium and phosphorus (5, 6, 12, 13). However, it was possible to demonstrate histological changes in teeth due to lack of vitamin D on a high calcium-low phosphorus diet analogous to that found in bone (31–37).

Table IX

Relation of Enamel, Dentin PO_4:$2CO_3$, and Blood Serum PO_4:CO_3 (Mean Values)

Group	Reference	B	D	C	B	D	C
Vitamin D		−	−	−	+	+	+
Experimental period, 30 days							
Serum × 1000	148	25.1	58.6	101	39.4	77.5	108
Enamel × 100	325	208	265	460	243	300	368
Dentin × 100	656	440	515	543	460	541	598
Experimental period, 45 days							
Serum × 1000	104	24.1	37.2	61.5	42.7	59.9	67.0
Enamel × 100	334	371	592	772	362	567	694
Dentin × 100	488	547	687	931	555	727	797

PO_4:CO_3 ratios in enamel are in all cases lower than in dentin. The difference was most marked in the high calcium-low phosphorus group (Diet B) and least in the low calcium-high phosphorus group (Diet C). Logan reported (26) that human enamel has a lower carbonate content than human dentin, whereas the carbonate contents of dog enamel and dentin were nearly equal. Bremer (38) found more carbonate in dog dentin than in dog enamel. He also found differences in regions of the enamel and dentin of a single human tooth. Armstrong and Brekhus (24) reported higher PO_4:CO_3 ratios in the enamel than in the dentin of human teeth. In view of the above findings with rats, one can point out that *the differences in composition between enamel and dentin may be due to a particular diet rather than to species differentiation.* That more than one type of composition of dentin exists in a given species is indicated by the findings of Bale *et al.* (39). They found that on heating dentin to 900° some samples gave the apatite pattern, while others had the β-$Ca_3(PO_4)_2$ lattice. Not until we understand the influence of diet in a given species can we discuss the differences between enamel and dentin as characteristic of various species.

The variations in PO_4:$2CO_3$ ratios in the different groups, which is a measure of n in the apatite formula, $[Ca_3(PO_4)_2]_n[CaCO_3]$, in these teeth, is far greater than those hitherto encountered in bone. In enamel this is from 2.08 to 7.72 and in the dentin from 4.40 to 9.31. In bone the widest spread hitherto found has been 1.86 to 3.33 (6, 9, 40). The question arises as to whether with longer experimental periods (over 45 days) the spread of n would not be even greater.

The Ca:PO_4 ratios[2] of the enamel are distinctly higher than those of the corresponding dentin in all dietary groups (see Tables VI to VIII). Such differences were not found in the reported analyses of human teeth, most of which are given in Armstrong's review (41), nor in dog teeth (26). The ratios of residual total base to $2PO_4$ (Table VIII) are all higher than 1.50 for enamel and less than 1.50 for dentin. Our x-ray diffraction studies[3] indicate that apatite is the main lattice in both the enamel and dentin of such teeth. This would imply that in enamel for the apatite formula, $[Ca_3(PO_4)_2]_n[CaX]$, X must have some other radical in addition to carbonate, which may be hydroxyl or possibly citrate (28) or fluoride. For dentin, X is some phosphate, probably $HPO_4^=$ (6, 29). We hope that x-ray diffraction studies, which are in progress, may throw further light on this subject.

While Ca:PO_4 ratios in serum undergo wide variation, no such wide variations are found for Ca:PO_4 or residual Ca:PO_4 in enamel and dentin. On the whole, the ratios in Group B were higher than those in Group C. In this connection one may note that the variations in Ca:PO_4 ratios in bone are relatively slight and were significant only between Groups B and C in the absence of vitamin D (6). It may be concluded that the PO_4:CO_3 ratios in bone and teeth are characterized by large variations, whereas the Ca:PO_4 ratios are characterized by slight variations.

---

[2] It must be emphasized that all through these analyses a ground-up bone sample was periodically analyzed with approximately the same amounts of solid and by the same methods as in the case of enamel and dentin. In each instance we obtained the same residual Ca:PO_4 ratio, which was between 1.51 and 1.52. The theoretical residual Ca:PO_4 ratio is 1.50 for $[Ca_3(PO_4)_2]_n[CaCO_3]$. Thus the changes in Ca:PO_4 and residual Ca:PO_4 ratios must be taken as valid.

[3] Sobel, A. E., Hanok, A., Kirshner, H., and Fankuchen, I., unpublished experiments.

In Table X, data from Experiments 112 and 117 of Logan and Taylor were recalculated (42). It may be noted that for a 33 per cent increase in the $PO_4:CO_3$ ratio of the initial liquid composition the ratio of the solid increased 41 per cent. For a 33 per cent increase in the $Ca:PO_4$ ratio of the initial liquid, the ratio in the solid increased 3 per cent, and the residual $Ca:PO_4$ ratio underwent no change at all. These studies are fragmentary and by no means conclusive; nevertheless they may be taken as clues explaining the relative constancy of $Ca:PO_4$ ratios in teeth and bone and the wide variations in the $PO_4:CO_3$ ratios. A systematic investigation of the inorganic precipitates formed in the presence of various amounts of Ca, PO_4, and CO_3 ions is needed to understand fully the relationships found between the composition of teeth and bone and blood serum.

TABLE X

$PO_4:2CO_3$ and $Ca:PO_4$ Ratios of Liquid and Solid (*Calculated from Data of Logan and Taylor* (*42*))

	$PO_4:2CO_3$		$Ca:PO_4$	
Experiment No., Logan and Taylor (42)	112	117	112	117
	molar ratio	*molar ratio*	*molar ratio*	*molar ratio*
Liquid initially	2.39	1.79	1.00	1.33
" at end of 20 days	1.00	0.17	0.0035	0.0094
Ppt. at end of 20 days	4.71	3.35	1.54	1.59
			1.44*	1.44*

* Residual $Ca:PO_4$ ratio.

The relationships established in these studies were found in the continuously growing incisors of rats. The question naturally arises as to whether molars and incisors of species which do not have continuous growth would behave in a similar fashion. The full answer cannot be given at present owing to lack of sufficient evidence. Experiments with isotopes indicate a turnover in even fully erupted molars (11–13). It is our contention that during the period of most active calcification of molars (or of incisors that do not grow continuously) the same laws must essentially hold true. As permanent teeth become more completely calcified, equilibrium with the body fluids slows up to such an extent that only minor changes can be observed. It is our hope that by controlling the diet of a species that has secondary dentition during the period of active calcification of the secondary teeth we will be able to put the above contention to experimental test.

An exact *quantitative relationship* between the composition of blood and teeth cannot be expressed at present, since this would involve a knowledge of the activities of the tooth-forming ions at the site of deposition. It would also require knowledge of the exact relationship between composition of the solid and the ionic activities of the ions that form the solid. Such knowledge is not yet available. These experiments, however, do indicate that the composition of serum is one of the controlling factors. Another factor is one which affects diffusion to the site of deposition. Vitamin D and age may play a part in the permeability of the membrane interposed between blood serum and growing teeth. Various "local factors" may also be instrumental in changing the composition of the fluid. Enzymatic factors have been shown to play a part in calcification of bone (43–45). Experiments on calcification of bone *in vitro* have shown that a minimal Ca × P product is required for deposition (46). This minimal Ca × P product was lowest in embryonic rabbit bone (47). In rachitic animals, a higher product was necessary for new calcification, and the more prolonged the rachitic period the higher the product required (48). In rickets due to beryllium and strontium the required product is much higher (49, 50). The experiments with rickets due to strontium indicate a reversible injury to a "local factor," enzymatic in nature and other than phosphatase. The more recent experiments of Gutman *et al.* (45) indicate that the enzyme system for "phosphorylative glycolosis" is important in the calcification of bone cartilage. From this discussion, it is evident that the "local factors" must be considered in the deposition of lime salts. "Local factors" in themselves operate within the limits prescribed by physicochemical laws, because in each case a minimal Ca × P product is required. The composition of bone is a reflection of the composition of serum (6), and the same holds true for teeth, as shown in these experiments. As yet the validity of a minimal Ca × P product for a given tooth has not been experimentally proved, but in view of the changes in composition, which indicate a physicochemical mechanism, it is likely that the "local factors" in teeth require a minimal Ca × P product. That "local factors" do have an influence is indicated by the difference in composition between enamel and dentin. There should be no differences if only humoral factors are effective.

It is possible that in addition to the Ca and P content other components of the diet will have an effect on the composition of the tooth. This may happen either by affecting the concentration or the diffusion of Ca, PO_4, and CO_3 across the body membranes or else by altering the nature of the "local factors." There is some evidence that dietary nutrients must play a part in the calcification of teeth and probably have a rôle in affecting composition. The importance of vitamins A and C for the proper development of the organic matrix and that of vitamin D for proper calcification have been repeatedly emphasized. The subject has been reviewed by Armstrong (51). Pincus has shown that an essential amino acid, tyrosine, is present in appreciable amounts in enamel protein (52). The diets which were used in these experiments are essentially modified rickets-producing diets and are not complete because, by adding vitamin D and sufficient optimal amounts of Ca and P, the rate of growth is still not that of the stock diet. It has been pointed out that rickets-producing diets have inadequate amounts of lysine (53). It is quite possible that on a different diet, for example one rich in all the essential amino acids, the composition of the teeth would have been different, since both the humoral and local factors might have been influenced. It is our contention, however, that a relationship between the $PO_4:CO_3$ ratios of serum and enamel and dentin would still exist, though possibly shifted to a higher or lower level. These studies serve to introduce the concept of the variability of the composition of the inorganic portion of teeth in relation to blood serum and diet, and will be continued. All of the factors that influence the composition of teeth merit serious attention.

The factors that influence the composition of enamel and dentin are likely to be of fundamental importance in understanding the problem of dental caries. Reports by Logan and Taylor (54) and by Dallemagne *et al.* (55, 56), indicate that the carbonate content of bone salt can be preferentially dissolved by dilute hydrochloric or citric acids. "Hence, bone salts are a simple physical complex of phosphate and carbonate. If we were dealing with carbonato-apatite, the two constituents...could not possibly be separated by using the same chemical methods" (56). From the fact that the acid-extracted bone residue before heating gave the apatite pattern and after ignition at 900° gave the β-$Ca_3(PO_4)_2$ pattern, they concluded that the main structure is α-$Ca_3(PO_4)_2 \cdot 2H_2O$ admixed with $CaCO_3$. When the two are heated together at 900°, apatite is obtained (56). The theoretical conclusions may be questioned, since treatment with acids (which remove the carbonate) may have also caused production of $CaHPO_4$. It has already been established that hydroxyapatite when ignited in the presence of $CaHPO_4$ is converted to β-$Ca_3(PO_4)_2$ (29). Regardless of the full validity of the theoretical conclusions, the important fact remains that the carbonate portions of bone salts can be dissolved under conditions that leave the phosphate practically intact. *If this holds true for teeth, it would indicate that teeth high in carbonate are more soluble in dilute acids than teeth low in carbonate, and are therefore more susceptible to at least one type of caries.*

Dallemagne *et al.* (56, 57) report, on the basis of optical methods only, that the mineral fraction of dentin and cementum from teeth is the same as that of bone. Enamel prisms consist of real carbonatoapatite. The interprismatic substance consists of organic matter impregnated mostly with α-$Ca_3(PO_4)_2 \cdot 2H_2O$. Dallemagne and Melon showed that the interprismatic substance (presumably α-$Ca_3(PO_4)_2 \cdot 2H_2O$) may be preferentially dissolved by salicylic acid (58). They did not exclude the possibility that the α-$Ca_3(PO_4)_2$ is, in turn, admixed with $CaCO_3$ to varying proportions. Thus one may envision a portion of the carbonate as the Achilles heel of the tooth, while that portion that goes into the carbonatoapatite is the resistant portion of the tooth. Further investigations are required to determine to what degree the diet influences the proportions of carbonatoapatite and adsorbed $CaCO_3$ in the tooth.

We hope that other investigations in progress will help to show the relationship of the present studies to caries.

SUMMARY

1. Experimental evidence is given for part of the hypothesis of the authors that *there is a relationship between the composition of teeth and the fluid from which the tooth salts precipitate and that the composition of this fluid in turn is related to the blood serum.* It was possible to show relationship between blood serum and the composition of the upper incisor of the white rat.
2. $PO_4:CO_3$ ratios of enamel and dentin are related to $PO_4:CO_3$ ratios in serum.
3. $PO_4:CO_3$ ratios of dentin are in all cases higher than those of corresponding enamels. The differences are not statistically significant when the $PO_4:CO_3$ ratios are highest.
4. In the formula $[Ca_3(PO_4)_2]_n[CaCO_3]$, n ranges from 2.08 to 7.72 for enamel and 4.40 to 9.31 for dentin in the various experimental groups. This is a far greater variation than has hitherto been observed in bone under comparable conditions.
5. The $Ca:PO_4$ ratios of enamel and dentin have undergone relatively small changes, whereas changes in the ratios for serum are relatively large.
6. The $Ca:PO_4$ ratios of enamel are in all groups significantly higher than those for the corresponding dentins.
7. Ratios for the residual total base to PO_4 and the residual Ca to PO_4 indicate that in the formula $[Ca_3(PO_4)_2]_n[CaX]$ for enamel X must have some other anion in addition to carbonate. In the dentin, X, in addition to carbonate, must have some phosphate, probably $HPO_4^=$.
8. It has been shown that the composition of the diet is reflected in the blood serum and that the composition of teeth (just as that of bone) is related to that of serum.
9. The density of minerals in neither enamel nor dentin is influenced by vitamin D. This is in contrast to the observations in bone, in which the density is significantly increased under the influence of vitamin D.
10. The implications of these findings for the understanding of the mech-

anism of calcification of teeth and the relation of composition to caries are discussed.

BIBLIOGRAPHY

1. Bethke, R. M., Kick, C. H., and Wilder, W., *J. Biol. Chem.*, **98**, 389 (1932).
2. Brown, H. B., Shohl, A. T., Chapman, E. E., Rose, C. S., and Saurwein, E. M., *J. Biol. Chem.*, **98**, 207 (1932).
3. Kramer, B., and Howland, J., *J. Nutr.*, **5**, 39 (1932).
4. Sobel, A. E., Yuska, H., Peters, D. D., and Kramer, B., *J. Biol. Chem.*, **132**, 239 (1940).
5. Sobel, A. E., Rockenmacher, M., and Kramer, B., *J. Biol. Chem.*, **158**, 475 (1945).
6. Sobel, A. E., Rockenmacher, M., and Kramer, B., *J. Biol. Chem.*, **159**, 159 (1945).
7. Taylor, N. W., and Sheard, C., *J. Biol. Chem.*, **81**, 479 (1929).
8. Roseberry, H. H., Hastings, A. B., and Morse, J. K., *J. Biol. Chem.*, **90**, 395 (1931).
9. Huggins, C., *Physiol. Rev.*, **17**, 119 (1937).
10. Deakins, M., and Burt, R. L., *J. Biol. Chem.*, **156**, 77 (1944).
11. Barnum, C. P., and Armstrong, W. D., *Am. J. Physiol.*, **135**, 2 (1942).
12. Greenberg, D. M., *J. Biol. Chem.*, **157**, 99 (1945).
13. Armstrong, W. D., and Barnum, C. P., *J. Biol. Chem.*, **172**, 199 (1948).
14. Bills, C. W., Honeywell, E. M., Wirick, A. M., and Nussmeier, M., *J. Biol. Chem.*, **90**, 619 (1931).
15. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61**, 523 (1924).
16. Sobel, A. E., and Sobel, B. A., *J. Biol. Chem.*, **129**, 721 (1939).
17. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
18. Sobel, A. E., *Ind. and Eng. Chem., Anal. Ed.*, **17**, 242 (1945).
19. Manly, R. S., and Hodge, H. C., *J. Dent. Res.*, **18**, 133 (1939).
20. Sobel, A. E., Rockenmacher, M., and Kramer, B., *J. Biol. Chem.*, **152**, 255 (1944).
21. McFayden, D. A., *J. Biol. Chem.*, **145**, 387 (1942).
22. Fisher, R. A., Statistical methods for research workers, Edinburgh, 6th edition, 128 (1936).
23. Bowes, J. H., and Murray, M. M., *Biochem. J.*, **29**, 2721 (1935).
24. Armstrong, W. D., and Brekhus, P. J., *J. Biol. Chem.*, **120**, 677 (1937).
25. Murray, M. M., and Bowes, J. H., *Brit. Dent. J.*, **61**, 473 (1936).
26. Logan, M. A., *J. Biol. Chem.*, **110**, 375 (1935).
27. Kirshner, H., Sobel, A. E., Hanok, A., and Fankuchen, I., Division of Biological Chemistry, American Chemical Society, 114th meeting, Washington, Aug. 30–Sept. 3 (1948).
28. Free, A. H., *J. Dent. Res.*, **22**, 477 (1943).
29. Hirschman, A., Sobel, A. E., Kramer, B., and Fankuchen, I., *J. Biol. Chem.*, **171**, 285 (1947).
30. Polskin, L. J., Kramer, B., and Sobel, A. E., *J. Nutr.*, **30**, 451 (1945).
31. Irving, J. T., *J. Dent. Res.*, **22**, 447 (1943).
32. Irving, J. T., *J. Physiol.*, **103**, 9 (1944).
33. Irving, J. T., *Nature*, **156**, 664 (1945).
34. Irving, J. T., *J. Physiol.*, **104**, 253 (1946).
35. Irving, J. T., and Nienbar, M. W., *J. Dent. Res.*, **25**, 327 (1946).
36. Weinman, J. P., and Schour, I., *Am. J. Path.*, **21**, 821 (1945).
37. Weinman, J. P., and Schour, I., *Am. J. Path.*, **21**, 1047 (1945).
38. Bremer, G., *Upsala Läkarefören. Förh.*, **44**, 219 (1938–39).

39. Bale, W. F., LeFevre, M. L., and Hodge, H. C., *Naturwissenschaften*, **24**, 636 (1936).
40. Logan, M., *Physiol. Rev.*, **20**, 522 (1940).
41. Armstrong, W. D., in Annual review of biochemistry, Stanford University, **11**, 441 (1942).
42. Logan, M., and Taylor, H. L., *J. Biol. Chem.*, **125**, 377 (1938).
43. Robinson, R., The significance of phosphoric esters in metabolism, New York (1932).
44. Kay, H. D., *Physiol. Rev.*, **12**, 384 (1932).
45. Gutman, A. B., Warrick, F. B., and Gutman, E. B., *Science*, **95**, 461 (1942).
46. Shipley, P. G., Kramer, B., and Howland, J., *Biochem. J.*, **20**, 379 (1926).
47. Niven, J. S. F., and Robinson, R., *Biochem. J.*, **28**, 2237 (1934).
48. Rosenheim, A. H., *Biochem. J.*, **28**, 708 (1934).
49. Sobel, A. E., Cohen, J., and Kramer, B., *Biochem. J.*, **29**, 2640, 2646 (1935).
50. Sobel, A. E., Goldfarb, A. R., and Kramer, B., *J. Biol. Chem.*, **108**, 395 (1935).
51. Armstrong, W. C., *J. Dent. Res.*, **27**, 376 (1948).
52. Pincus, P., *Biochem. J.*, **42**, 219 (1948).
53. Francis, P. S., *J. Assn. Official Agr. Chem.*, **30**, 364 (1947).
54. Logan, M. A., and Taylor, H. L., *J. Biol. Chem.*, **125**, 391 (1938).
55. Dallemagne, M. J., and Brasseur, H., *Bull. Soc. roy. sc. Liége*, Nos. 8–9 (1942).
56. Brasseur, H., Dallemagne, M. J., and Melon, J., *Nature*, **157**, 453 (1946).
57. Dallemagne, M. J., and Melon, J., *Bull. Soc. chim. biol.*, **27**, 85 (1945).
58. Dallemagne, M. J., and Melon, J., *Extrait Arch. biol.*, **57**, 1 (1946).

A METHOD OF ASSAY FOR CYTIDINE AND URIDINE BY MEANS OF A PYRIMIDINE-DEFICIENT STRAIN OF NEUROSPORA

By HUBERT S. LORING, GEORGE L. ORDWAY, AND JOHN G. PIERCE

(From the Department of Chemistry and the School of Medicine, Stanford University, California)

(Received for publication, June 1, 1948)

Knowledge of the composition of ribonucleic acid is based in part on elementary analyses and in part on analyses for the constituent purines, pyrimidines, easily hydrolyzed ribose, and phosphate. While free adenine and guanine are liberated by mild acid hydrolysis and can be determined by several methods, the same does not hold for the free pyrimidines. The latter are freed only after strong acid hydrolysis under conditions which have been demonstrated to convert cytosine in part to uracil (1). Since the pyrimidine nucleotides are produced by mild acid hydrolysis of ribonucleic acid and form relatively insoluble barium salts, this procedure has also been used in estimating their concentration. Analyses of the mixture of barium salts for amino nitrogen have given values corresponding to 47 per cent cytidylic acid and 53 per cent uridylic acid (2). The yield obtained, however, has been estimated to represent a maximum of only about 35 per cent of that expected from the statistical tetranucleotide theory (3). An examination of other evidence on which this theory of the structure of ribonucleic acid is based, as well as a consideration of more recent experimental data not in agreement with the tetranucleotide theory (4, 5), has stressed the need for a more quantitative method of analysis for the pyrimidine components of ribonucleic acid. The production of a *Neurospora* mutant, No. 1298, which requires the pyrimidine ribonucleosides or ribonucleotides for growth has made possible such a method (6). The present paper is concerned with the use of this mutant strain for the assay of the pyrimidine ribonucleosides, with a method of fractionation by which cytidine can be separated nearly quantitatively from uridine in known mixtures, and with the preliminary results obtained by this method as applied to certain nucleic acid hydrolysates.

EXPERIMENTAL

The method of culturing mutant 1298 and its growth characteristics on different pyrimidine compounds have been presented previously (6). A comparison of the two methods of determining growth rate, namely rate of progression of mycelium on solid medium and dry weight in liquid culture, showed that a higher degree of reproducibility could be obtained with the latter procedure, provided the flasks were not shaken during the growth period.

Growth Responses to Cytidine and Uridine—The growth responses to cytidine and to uridine were determined by adding varying amounts of each in a 1 ml. volume to 25 ml. of basal medium in 125 ml. Erlenmeyer flasks. The flasks were autoclaved, inoculated as previously described (6), and placed in an incubator at 25° ± 0.2° for 72 hours. The contents were poured into a Petri dish, and the mycelium pad picked up with forceps, squeezed between the fingers, rinsed twice with a small stream of distilled water, and allowed to dry between the folds of a paper towel for 3 to 4 hours at 60–80°. The dry weights of mycelium obtained in this way for different amounts of cytidine and uridine are shown graphically in Fig. 1.

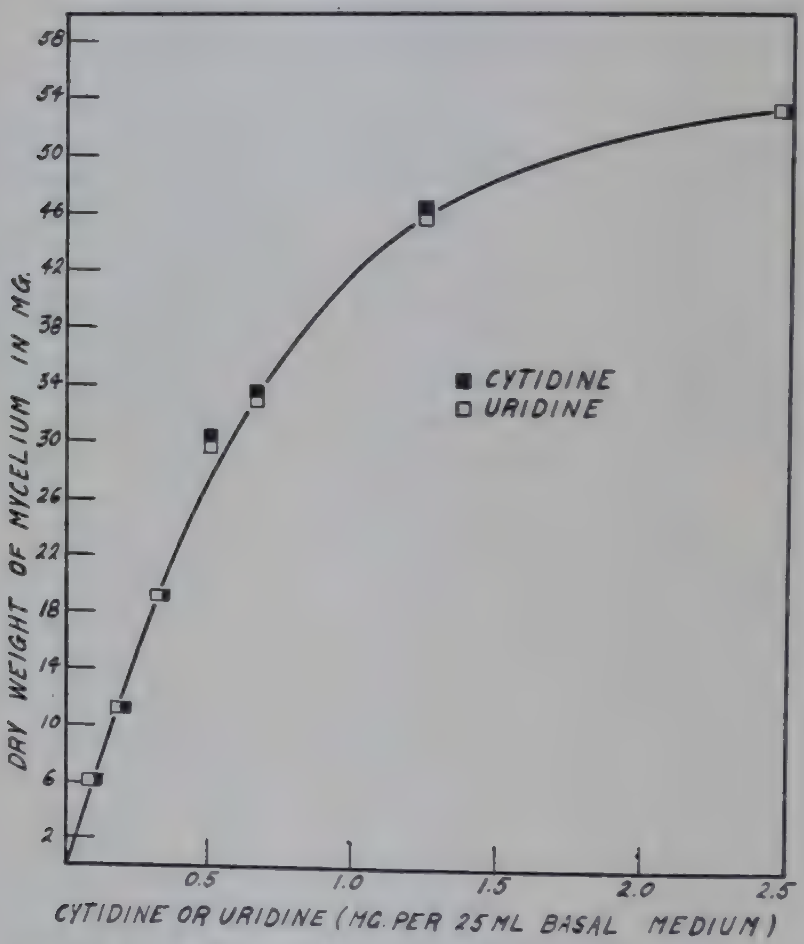


Fig. 1. Growth curve of mutant strain 1298 on uridine (□) and cytidine (■)

The growth responses to the two compounds are the same within experimental error when the points on the curves are determined in triplicate. Under conditions up to about half maximum growth a relatively high degree of precision is obtained. The standard deviations in five replicate determinations each at average mycelium weights of 30.7 and 15.5 mg. were 0.6 and 0.4 mg., respectively, corresponding to values for cytidine of 0.58 ± 0.02 mg. and 0.24 ± 0.01 mg. It is evident from Fig. 1 that the greatest relative growth response was found when amounts of supplement giving less than one-half maximum growth were used. As in other *Neurospora* assays the growth curve is approximately linear up to a dry weight of about 20 mg. The relation between weight of mycelium and cytidine or uridine over this range is given with an accuracy of from 5 to 10 per cent by the equation, cytidine or uridine in mg. = 0.0165 × weight of mycelium in mg.

The volume of solution containing the growth supplement may be increased up to about 5 ml. per 25 ml. of basal medium without significantly affecting the amount of growth. Further dilution, however, probably causes slightly less growth. Of greater importance is the age of the culture used for inoculation. Curves similar to that shown were obtained provided the culture was less than about 5 weeks old. Those older than 5 weeks gave slightly less growth at the different concentrations of supplement.

A procedure similar to that used for obtaining the standard curve is employed for the assay of an unknown solution. Since growth of the mold is inhibited by solutions that are too acid or too alkaline, it is necessary to adjust the pH of the basal medium containing the unknown solution before autoclaving. A convenient method for doing this is to use bromocresol green as an inside indicator and to adjust the pH to 4.5 with acid or alkali as needed (7). Sodium chloride or sodium sulfate in amounts up to about 100 mg. per 25 ml. of basal medium are not inhibitory. The mold, however, is highly sensitive to traces of phosphotungstic acid used for the fractionation of the purine and pyrimidine components or to traces of heavy metals like lead or silver. It is also inhibited by traces of detergents, and glassware washed by a detergent solution should be acid-rinsed before using. It is usually possible to tell whether or not a partial inhibition is present from the type of mold growth obtained. While a single, small mycelium mat is present at lower concentrations of growth supplement in the absence of inhibition, many small foci of growth are sometimes found if an inhibitory substance is present. In other cases partial inhibition is indicated by the slimy nature of the mycelium pad. When inhibition is suspected, it is advisable to carry out additional assays with smaller and larger amounts of the unknown solution. If an inhibitory substance is present, the larger volume of the unknown solution usually results in a relatively greater degree of inhibition, while the opposite is true if less of the unknown is used. It is desirable, therefore, to show that the same value for cytidine or uridine is obtained at different levels of growth.

Cytidine Phosphotungstate—As cytidine and uridine occur together in ribonucleic acid, the use of mutant 1298 for the assay of either compound depends on the success with which they can be separated from each other. As cytidine possesses a basic amino group, a number of acids which form relatively insoluble salts with organic bases were investigated to determine their usefulness as possible precipitating agents. Of the five acids studied, picric, picrolonic, nitranilic, flavianic, and phospho-12-tungstic (8), the last gave the most insoluble salt. When sufficient phosphotungstic acid was added to a warm cytidine solution in 1 N hydrochloric acid to give a 2 to 3 mg. per ml. excess, beautifully crystalline prismatic needles of cytidine phosphotungstate separated as shown in Fig. 2. A sample recrystallized once from 1 N hydrochloric acid and air-dried gave the following analyses:[1] H_2O 8.92, $H_3PO_4 \cdot 12WO_3$ 78.7, C 5.41, H 1.64 per cent. These compare favorably with theoretical values of H_2O 8.78, $H_3PO_4 \cdot 12WO_3$ 78.2, C 5.85, and H 1.72 per cent for $(C_9H_{12}O_5N_3)_2 \cdot H_3PO_4 \cdot 12WO_3 \cdot 18H_2O$. Under similar conditions uridine gave no precipitate with phosphotungstic acid.

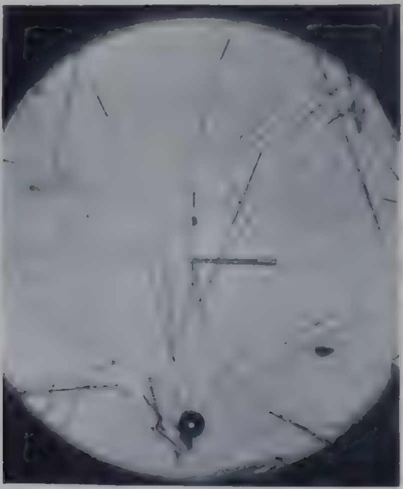


FIG. 2. Cytidine phosphotungstate (magnification about 100 ×)

Solubility of Cytidine Phosphotungstate—The solubility of cytidine phosphotungstate in 1 N hydrochloric acid at 0° was determined from the supersaturated side by precipitating cytidine in the presence of a 2 to 3 mg. per ml. excess of phosphotungstic acid. The filtrate was treated with excess ammonium chloride in 1 N hydrochloric acid to remove the excess phosphotungstic acid,[2] and the filtrate was assayed for cytidine with mutant

---

[1] C and H analyses by Laboratory of Microchemistry, 366 Fifth Avenue, New York.

[2] The solubility of ammonium phosphotungstate in 1 N hydrochloric acid is less than 0.002 mg. of nitrogen per ml. as shown by the fact that such a concentration of ammonium sulfate nitrogen gives a precipitate with phosphotungstic acid under these conditions.

1298. An average value of 0.08 mg. of cytidine per ml. of the original cytidine phosphotungstate filtrate was found, indicating a nearly quantitative precipitation of cytidine under these conditions.

Fractionation of Cytidine and Uridine—The following experiment illustrates the degree of success with which cytidine was separated from an equal amount of uridine. To 20 ml. of warm 1 N hydrochloric acid containing 10 mg. each of cytidine and uridine, 80 mg. of phosphotungstic acid dissolved in 5 ml. of 1 N acid were added. A crystalline precipitate of cytidine phosphotungstate separated. The suspension was allowed to stand in ice in the cold room overnight and was filtered in the cold by gravity. To 20 ml. of this filtrate 75 mg. of ammonium chloride dissolved in 0.5 ml. of 1 N acid were added. After standing overnight in the cold room, the suspension containing ammonium phosphotungstate was filtered and 1 ml. aliquots of the filtrate were assayed for activity with the mold. An average dry weight of mycelium corresponding to 0.51 mg. of uridine was found as compared to the calculated value of 0.47 mg. if the value for the solubility of cytidine phosphotungstate given above, is used and it is assumed that no uridine was precipitated. Other experiments gave similar results indicating that the two compounds could be fractionated by this procedure from relatively dilute solutions with a high degree of success.

Hydrolysis and Assay of Ribonucleic Acid—The original procedure attempted for the hydrolysis of ribonucleic acid to ribonucleosides was that given by Jones (p. 112 (9)) for the preparation of ribonucleosides. Assay of a hydrolysate obtained by this method from a sample of purified ammonium nucleate (4) gave a value of 15.2 per cent total pyrimidine nucleosides as compared to that of 34 per cent expected for a statistical tetranucleotide composed of equimolar quantities of adenylic, guanylic, cytidylic, and uridylic acids and containing 10 per cent water, the approximate amount found in most samples of yeast nucleic acid. The surprisingly low value suggested an incomplete hydrolysis and further experiments were carried out to determine the maximum yield of pyrimidine nucleosides which could be obtained after heating for various lengths of time up to 8 hours. Analyses for inorganic and total phosphate were made as well after the 4 and 8 hour periods.

Two concentrations of ammonium nucleate in 2.5 per cent ammonia, namely 20 per cent, the concentration used by Jones, and 2.25 per cent, were employed in the hydrolysis experiments. In the first case 100 mg. samples in 0.5 ml. and in the second 22.5 mg. samples in 1 ml. in small sealed Pyrex tubes were heated in an oil bath at 140–150°. In the first experiment tubes were taken from the bath at the end of 1, 2, 3, and 4 hours and in the second at the end of 4 and 8 hours, and their contents were removed, diluted with distilled water, and assayed for activity. The average values expressed as per cent total pyrimidine nucleoside and as molar ratios of pyrimidine nucleoside to total phosphate are shown in Table I in comparison with the ratio expected from a statistical tetranucleotide. It may be seen that values corresponding to about 20 per cent pyrimidine nucleosides and a ratio of 0.36 moles of pyrimidine nucleoside per mole of phosphorus were obtained with the more concentrated as well as the less concentrated nucleic acid hydrolysates after 3, 4, and 8 hours. In comparison with the value calculated from the tetranucleotide theory, the largest value found corresponds to about 78 per cent of that expected. The difference between the 4 and the 8 hour values in the less concentrated sample, 0.39 as compared to 0.36, is not highly significant in terms of the precision of the assay procedure, but the results indicate, in agreement with other experiments on the stability of the pyrimidine nucleosides in weak alkali, that these compounds are destroyed to a slight extent during the longer period of heating. If it is assumed that a similar loss took place during the first 4 hours of hydrolysis, the highest value for the ratio of pyrimidine nucleosides to phosphorus becomes 0.42 in comparison with the theoretical of 0.50. Inorganic phosphate after 4 and 8 hours corresponded to 94 and 102 per cent respectively of the total phosphorus, showing that the nucleic acid was not completely hydrolyzed to the nucleoside stage under the conditions used until after 4 hours.

TABLE I

Total Pyrimidine Nucleosides and Inorganic Phosphate Found after Heating Yeast Ribonucleic Acid in 2.5 Per Cent Ammonia at 140–150° for Varying Lengths of Time

Experiment No.	Length of time heated	Total pyrimidine nucleosides	Inorganic phosphate	Pyrimidine nucleoside found / Total phosphate
	hrs.	*per cent*	*per cent*	*mole per mole P*
1	1	2.4		0.043
	2	15.1		0.27
	3	20.0		0.36
	4	21.6		0.38
2	4	21.7	6.72	0.39
	8	20.0	7.3	0.36
Statistical tetranucleotide...				0.50

In Experiment 1 the nucleic acid concentration was 20 per cent and in Experiment 2, 2.25 per cent. Phosphorus content of ammonium nucleate used = 7.14 per cent.

As adenosine is present in the hydrolysates mentioned above and is known to inhibit the utilization of cytidine and uridine by this strain of *Neurospora* if present in sufficient concentration (10), the low values for total pyrimidine nucleosides might be explained on this basis. An examination of published data (4) for the ratios of guanine and adenine to phosphorus in the same nucleic acid preparation, however, revealed that the ratio of adenosine found previously to the pyrimidine nucleoside content given in this paper was not sufficiently high to result in inhibition provided that this sample of nucleic acid contained approximately equal quantities of both cytidine and uridine.

A few experiments were performed in which aliquots of hydrolysates prepared after 4 and 8 hours of heating were fractionated by phosphotungstic acid to separate the purine components and cytidine from the uridine present. Assays of the resulting filtrates gave values for uridine very nearly one-half those found for both cytidine and uridine before fractionation. These results are therefore in agreement with the older data (2) that cytidine and uridine are present in equimolar quantities in yeast ribonucleic acid. A number of fractionation experiments on larger samples and on other yeast nucleic acid preparations which will be published in a subsequent paper[3] led to the same conclusion.

SUMMARY

A method is described for the determination of cytidine and uridine, by means of the pyrimidine-deficient mutant strain of *Neurospora*, No. 1298. The method permits the determination of these pyrimidine ribonucleosides over the concentration range from about 0.05 mg. to about 0.6 mg. in a volume of 5 ml. or less with a standard deviation of about 5 per cent. Cytidine forms a relatively insoluble, crystalline salt with phospho-12-tungstic acid, $H_3PO_4 \cdot 12WO_3 \cdot 7H_2O$, having a solubility in 1 N hydrochloric acid of 0.08 mg. per ml. (as cytidine) and can be separated from uridine in known mixtures with a high degree of success. The assay of hydrolysates prepared by heating samples of purified ammonium nucleate from yeast with 2.5 per cent ammonia at 140–150° for varying periods of time shows that the concentration of pyrimidine nucleosides reaches a maximum after 4 hours. The amount found for this preparation was 23.4 per cent or, expressed in relation to its phosphorus content, 0.42 mole of pyrimidine nucleoside per mole of phosphorus. Fractionation experiments with phosphotungstic acid showed that equal amounts of cytidine and uridine were present.

BIBLIOGRAPHY

1. Osborne, T. B., and Harris, I. F., *Z. physiol. Chem.*, **36**, 85 (1902).
2. Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, **44**, 1027 (1911).
3. Hagenguth, K., *Z. physiol. Chem.*, **239**, 127 (1936).

---

[3] Loring, H. S., and Ploeser, J. M., unpublished work.

4. Loring, H. S., Carpenter, F. H., and Roll, P. M., *J. Biol. Chem.*, **169**, 601 (1947).
5. Loring, H. S., Ordway, G. L., Roll, P. M., and Pierce, J. G., *Federation Proc.*, **6**, 510 (1947).
6. Loring, H. S., and Pierce, J. G., *J. Biol. Chem.*, **153**, 61 (1944).
7. Ryan, F. J., and Brand, E., *J. Biol. Chem.*, **154**, 161 (1944).
8. Van Slyke, D. D., Hiller, A., and Dillon, R. T., J. Biol. Chem., **146**, 137 (1942).
9. Jones, W., Nucleic acids, Monographs on biochemistry, London and New York, 2nd edition (1920).
10. Pierce, J. G., and Loring, H. S., *Federation Proc.*, **6**, 514 (1947); *J. Biol. Chem.*, **176**, 1131 (1948).

PURINE AND PYRIMIDINE ANTAGONISM IN A PYRIMIDINE-DEFICIENT MUTANT OF NEUROSPORA

By JOHN G. PIERCE* AND HUBERT S. LORING

(From the Department of Chemistry and the School of Medicine, Stanford University, California)

(Received for publication, June 1, 1948)

Inhibitions of the growth of organisms by closely related structural analogues of several water-soluble vitamins are well known. Similar types of antagonism in the case of purine and pyrimidine metabolites are those between the purine bases, adenine and guanine, and benzimidazole (1) and between barbituric acid and uracil in the growth of *Staphylococcus aureus* (2). In such cases the antagonism is usually considered to be due to competition between the analogue and the metabolite in enzyme systems where the normal metabolite is involved.

Antagonisms between two structurally related, naturally occurring substances are less common but have been demonstrated to exist between amino acids of similar structure (3, 4) and between thiamine and pyridoxine (5). A most striking relationship between normal metabolites is that reported by Raska (6) in which pellagra was produced experimentally in dogs when adenine alone or in conjunction with phosphate was fed in daily doses of 400 to 500 mg. An explanation similar to that mentioned above for the antagonism between metabolites and structural analogues not occurring naturally has also been offered for the closely related natural substances.

The present study is concerned with the inhibition of growth of the pyrimidine-deficient *Neurospora* mutant, No. 1298, by the naturally occurring purine ribonucleotides and ribonucleosides. This experimentally produced strain, unlike the wild type, is unable to synthesize the pyrimidine ribonucleosides on a medium containing inorganic salts, carbohydrate, and biotin. Normal growth takes place, however, when the medium is supplemented with either cytidine or uridine or the corresponding nucleotides (7). It has been found that adenosine and adenosine-3-phosphate (yeast adenylic acid) inhibit the utilization of the pyrimidine compounds to a varying degree. An amount of adenine nucleoside which is sufficient to inhibit growth completely on the quantity of cytidine used has no inhibitory effect on an equivalent amount of uridine. The addition of an equimolar amount of uridine to a mixture of cytidine and adenosine in which no growth takes place results in the elimination of the antagonism. In contrast to the effect of adenosine and adenylic acid on this mutant strain of *Neurospora*, adenine shows no inhibitory properties at comparable concentrations. A similar inhibitory effect on the utilization of the pyrimidine nucleosides was found for guanosine and guanylic acid, but larger amounts of these compounds were required to produce inhibition under the same conditions. Guanine like adenine failed to cause inhibition at moderate concentrations.

---

* American Chemical Society Postdoctorate Fellow. Present address, Department of Biochemistry, Cornell University Medical College, New York City.

EXPERIMENTAL

The growth response of the mold to various concentrations of supplements and inhibitors as compared to that in the absence of inhibitor was determined from the dry weight of mycelium produced in liquid culture after incubation for 3 days at 25°. The composition of the basal medium, the method of inoculation, and the determination of the weight of mycelium were the same as previously described (7, 8). The concentrations of the pyrimidine derivatives used in the determination of the inhibitory effects of the purine compounds were those which produced an approximately half maximum growth of the mold. In this range an amount of mold which can be readily weighed is obtained, and growth response is most sensitive to small changes in the concentration of added supplement. The growth of the mold was found from the average value of determinations made in triplicate.

Uridylic acid, in the form of the diammonium salt, cytidylic acid, uridine,[1] and guanine[2] were prepared by methods devised in this laboratory (9). Guanosine was isolated from yeast nucleic acid as described by Levene (10). The cytidine, guanylic acid, adenosine, and adenosine-3-phosphate were commercial samples.[3]

Antagonism by Adenosine and Adenosine-3-phosphate—The effect of adenine, adenosine, and adenosine-3-phosphate on the growth activity of cytidine, uridine, cytidylic acid, and uridylic acid was determined by adding increasing amounts of each purine compound to the basal medium supplemented with a constant amount of growth factor. The amount of cytidine or uridine used was 0.5 mg. per 25 ml. of basal medium. The growth of the mold in the presence of varying amounts of adenine, adenosine, and adenosine-3-phosphate was determined and plotted as the percentage of growth obtained in the absence of inhibition. The data for cytidine and the three purine compounds and for uridine and adenosine are shown in Fig. 1. It can be seen that adenosine was twice as inhibitory of cytidine activity as was adenosine-3-phosphate. When uridine was used as the growth factor, approximately five times as much adenosine was required to produce the same degree of inhibition. Free adenine failed to inhibit the growth of the mold on cytidine at a concentration equivalent to 0.6 mg. of adenosine and indeed was slightly stimulatory at some concentrations. Similarly, no significant inhibition of uridine in the presence of adenine was observed at a concentration equivalent to 4.0 mg. of adenosine.

---

[1] Loring, H. S., and Ploeser, J. McT., unpublished work.

[2] Loring, H. S., and Ali, S. A., unpublished work.

[3] Cytidine and guanylic acid were kindly provided by the National Biochemical Corporation, New York. Adenosine and adenosine-3-phosphate were obtained from Schwarz Laboratories, Inc., New York.

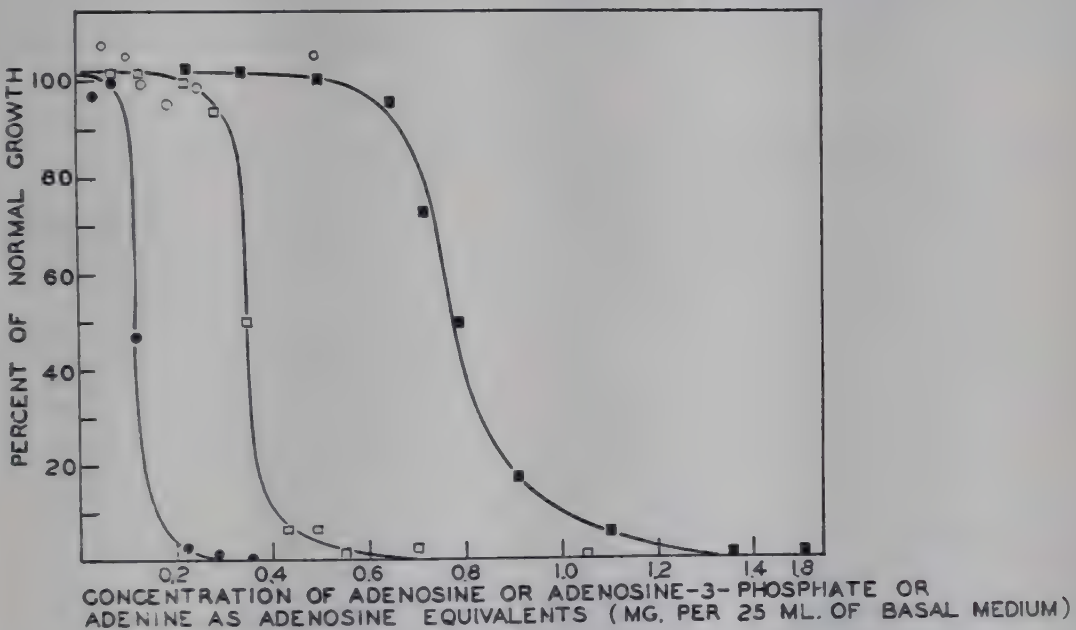


FIG. 1. The effect of adenine, adenosine, and adenosine-3-phosphate expressed as adenosine or adenosine equivalents on the growth of *Neurospora* mutant No. 1298; ○ adenine, ● adenosine, and □ adenosine-3-phosphate in the presence of 0.5 mg. of cytidine; ■ adenosine in the presence of 0.5 mg. of uridine.

In the case of cytidylic acid and uridylic acid, the amounts employed to give about half maximum growth were 1 mg. of cytidylic acid and 1 mg. of diammonium uridylate per 25 ml. of basal medium. Addition of the three adenine compounds in similar amounts to those used for the pyrimidine nucleosides gave inhibition curves of the same type as those shown in Fig. 1. Free adenine as with cytidine and uridine did not affect the utilization of the pyrimidine ribonucleotides. The molar ratios of antagonist to metabolite to give 50 per cent inhibition in the case of the four pyrimidine compounds and adenosine and adenosine-3-phosphate were calculated from the respective inhibition curves and are given in Table I. Of the four pyrimidine compounds it may be seen that cytidylic acid was the most readily inhibited, the molar ratio of adenosine to cytidylic acid for 50 per cent inhibition being 0.13. In contrast to cytidylic acid, the inhibition ratio for adenosine and uridylic acid was 0.41. It is evident that adenosine-3-phosphate was less inhibitory in all cases than the corresponding nucleoside. Cytidine, like cytidylic acid, was more strongly inhibited than uridine, but each pyrimidine nucleoside was affected to a lesser degree by adenosine than was the corresponding nucleotide.

Antagonism by Guanosine and Guanosine-3-phosphate—An entirely analogous situation was found in the case of the guanine compounds. Guanosine and guanylic acid inhibited the utilization of each of the four pyrimidine nucleosides or nucleotides while guanine at comparable concentrations had no effect. The effect of guanosine and guanosine-3-phosphate on mold growth in the presence of 0.5 mg. of cytidine or uridine is shown in Fig. 2. It may be seen that approximately twice as much guanosine or guanylic acid was required to produce the same amount of inhibition as for the adenine compounds. Guanine in an amount equivalent to 10 mg. of guanosine per 25 ml. of basal medium did not affect the growth of the mold in the presence of 0.5 mg. of cytidine or uridine. The molar ratios of guanosine or guanylic acid to cytidine and uridine to produce 50 per cent inhibition are also shown in Table I.

TABLE I

Molar Ratios of Antagonist to Metabolite for 50 Per Cent Inhibition

Metabolite	Weight metabolite used per 25 ml. medium	Antagonist	$\frac{\text{Moles antagonist}}{\text{Moles metabolite}}$
	mg.		
Cytidylic acid	1	Adenylic acid	0.27
" "	1	Adenosine	0.13
Cytidine	0.5	Adenylic acid	0.60
"	0.5	Adenosine	0.24
Uridylic acid	0.86	Adenylic acid	0.6
" "	0.86	Adenosine	0.41
Uridine	0.5	Adenylic acid	3.2
"	0.5	Adenosine	1.4
Cytidine	0.5	Guanosine	0.68
"	0.5	Guanylic acid	1.29
Uridine	0.5	Guanosine	3.0
"	0.5	Guanylic acid	7.8

Antagonism in Mixtures of Pyrimidine Nucleosides and Nucleotides—The surprising difference in the ability of adenosine to inhibit growth on uridine as compared to cytidine suggested that the antagonism was involved to a different degree in the reactions concerned in the utilization of the two compounds. If the reaction inhibited was the deamination of cytidine to uridine rather than the utilization of cytidine *per se*, then it should be possible to eliminate the inhibition of cytidine by the addition of sufficient uridine to avoid the necessity of deamination. It was desirable, therefore, to determine the amount of uridine that would cause the reversal of the antagonism in an inhibitory mixture of cytidine and adenosine. A series of flasks containing 0.25 mg. of cytidine and 0.27 mg. of adenosine in 25 ml. of basal medium was supplemented with increasing amounts of uridine from 0.05 to 0.5 mg. The molar ratio of adenosine to cytidine was 1.0, which in the absence of uridine produces complete inhibition. The effect of the addition of the uridine is shown in Fig. 3, *A*, where the weight of mycelium found for each concentration of uridine was plotted against the total weight in mg. of cytidine and uridine used as the growth supplement. The curve showing the growth of the mold on either pyrimidine nucleoside in the absence of inhibitor is also presented. It may be seen that the growth-promoting properties of the mixtures were almost completely inhibited until the molar ratio of the cytidine to uridine approached 1. When the ratio reached 1, the inhibition was strikingly eliminated, and as more uridine was added, the amount of growth was approximately that found with either cytidine or uridine when no inhibitor was present. As shown in the cytidine-adenosine curve in Fig. 1, 0.27 mg. of adenosine in the

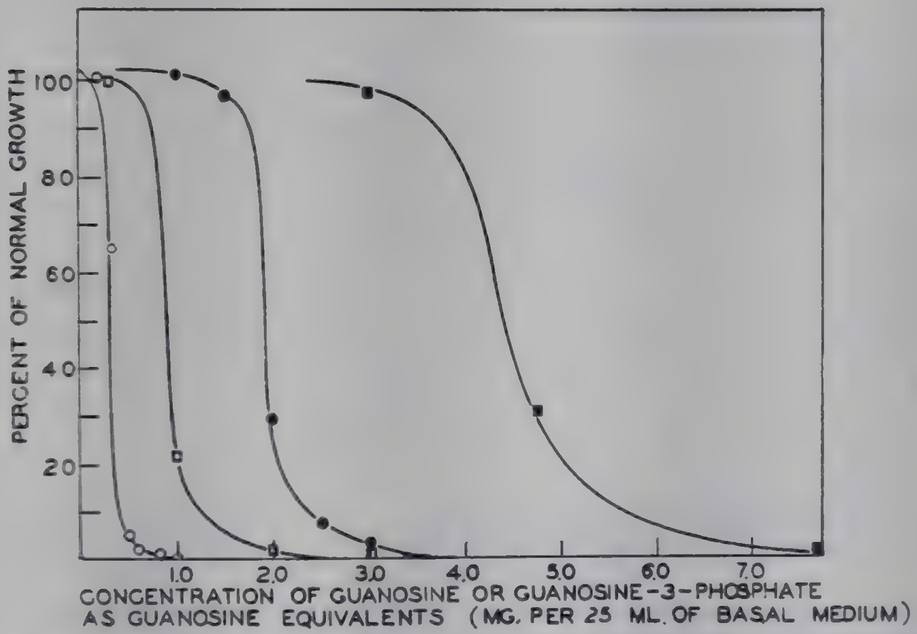


FIG. 2. The effect of guanosine or guanylic acid expressed as guanosine or guanosine equivalents on the growth of *Neurospora* mutant No. 1298; ○ guanosine, and □ guanylic acid in the presence of 0.5 mg. of cytidine; ● guanosine, and ■ guanylic acid in the presence of 0.5 mg. of uridine.

presence of 0.5 mg. of cytidine, a molar ratio of 0.5, gave 98 per cent inhibition. Thus it is evident that the inhibition was a specific one and that the addition of an equivalent quantity of cytidine instead of uridine would not have overcome the effect of the adenosine.

An experiment performed with 0.4 mg. of cytidylic acid and 0.18 mg. of adenosine, an amount giving complete inhibition, gave a similar elimination of antagonism when increasing amounts of uridylic acid were added. Inhibition was nearly 100 per cent when the ratio of uridylic acid to cytidylic acid was less than 1, but, when the ratio became 1, inhibition no longer was observed. In this case when an additional equivalent of cytidylic acid was added to the 0.4 mg. of cytidylic acid-0.18 mg. of adenosine mixture, the amount of growth corresponded to about 95 per cent inhibition.

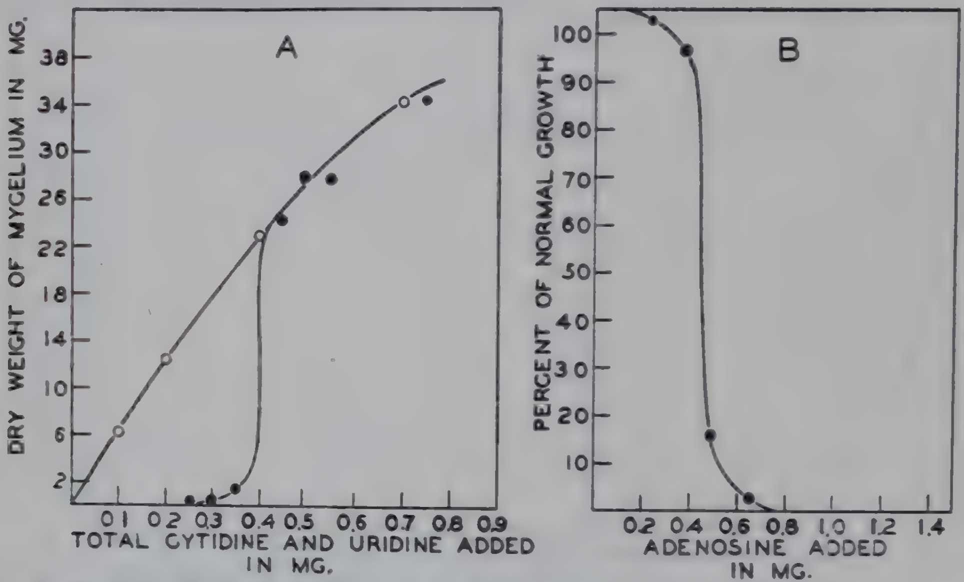


FIG. 3, *A*. The effect of uridine on an inhibitory mixture of 0.25 mg. of cytidine and 0.27 mg. of adenosine in comparison with the normal growth curve on cytidine or uridine; ○ normal growth curve on cytidine or uridine, ● growth curve on cytidine-adenosine mixture with varying amounts of uridine.

FIG. 3, *B*. The effect of adenosine on an equimolar mixture of 0.25 mg. of cytidine and 0.25 mg. of uridine.

In an attempt to reverse the guanosine inhibition of cytidine, increasing amounts of uridine were added to a mixture of cytidine and guanosine which is completely inhibitory (0.25 mg. of cytidine and 0.5 mg. of guanosine; *cf.* Fig. 2). The antagonism was also eliminated in this case by the addition of an equimolar amount of uridine, 0.25 mg. of the latter permitting growth equal to 82 per cent of that expected from either 0.5 mg. of cytidine or the same quantity of uridine with no inhibitor added.

The striking elimination of the inhibition of cytidine by an equimolar amount of uridine provided evidence that the antagonism of adenosine was concerned in a highly specific way with the deamination of cytidine to uridine, possibly by inhibition of a cytidine deaminase present in the mold. A possible explanation of the antagonism for uridine alone could be the blocking of the reverse reaction, the amination of uridine to cytidine. A larger amount of adenosine would be required for the blocking of this reaction than for the deamination, as shown by the larger amounts required to inhibit growth on uridine. If this were the case, one would expect the amount of adenosine which inhibits the utilization of uridine alone to have no effect on an equimolar mixture of cytidine and uridine, both being available for growth.

To study this question an equimolar mixture of 0.25 mg. of cytidine and 0.25 mg. of uridine was supplemented with different amounts of adenosine from 0.23 to 1.4 mg., and the amount of mold growth determined in each case. The mycelium weights expressed as per cent of growth in the absence of inhibitor and the amounts of adenosine added are shown graphically in Fig. 3, *B*. When 0.27 mg. of adenosine was used, an equimolar mixture of all three components was present, and the growth of the mold was not inhibited, as was expected from the curve shown in Fig. 3, *A*. However, as the ratio of adenosine to cytidine and uridine was increased, mold growth was inhibited in a fashion similar to that found for uridine alone. Thus for 50 per cent inhibition, the same adenosine-uridine ratio of about 1.4 was found in this experiment where cytidine was present as with uridine and adenosine alone. The effect of adenosine on uridine is probably concerned, therefore, with the utilization of uridine for growth directly rather than with its conversion to cytidine.

To ascertain whether the inhibitory effects of adenosine and guanosine on a mixture of cytidine and uridine were additive, increasing amounts of guanosine from 0.2 mg. to 1.0 mg. were added to flasks containing 0.25 mg. of cytidine, 0.25 mg. of uridine, and 0.45 mg. of adenosine in 25 ml. of basal medium. This mixture allows about 30 per cent of the normal growth of the mold to take place; *cf.* Fig. 3, *B*. The addition of the guanosine resulted in further inhibition.

To demonstrate that the antagonism observed between adenosine and cytidine is a competitive one, *i.e.*, that a constant ratio of antagonist to metabolite will give the same degree of growth regardless of the actual concentration of metabolite present, flasks were set up containing 0.25, 0.50, 0.75, and 1.0 mg. of cytidine per 25 ml. of basal medium. To these were added 0.07, 0.14, 0.21, and 0.28 mg. of adenosine, respectively. The percentage of normal growth obtained (as calculated from the standard growth curve (8)) was approximately the same at each concentration level of antagonist and metabolite, *i.e.*, 8, 6, 9, and 7 per cent, respectively.

Although the antagonism between cytidine and adenosine was also demonstrated to exist in the pyrimidine-deficient mutant of *Neurospora* No. 263-1895-3a[4] (7), it could not be shown in the wild type which is able to synthesize its pyrimidine requirements. In determining this fact 5 mg. of adenosine were added to 25 ml. of basal medium, and the growth of the wild type measured in the usual way. The amount of growth did not differ significantly in the presence of adenosine from that found with the unsupplemented medium alone.

DISCUSSION

The *Neurospora* mutant No. 1298, unlike its wild type counterpart, fails to grow on the basal medium alone but grows when the medium is supplemented with either of the two pyrimidine ribonucleosides or ribonucleotides. As these compounds are essential constituents of ribonucleic acid, it is evident that the failure of growth in their absence is due to a deficiency in ribonucleic acid synthesis. Because normal growth is obtained with either cytidine or uridine or the corresponding nucleotides, it is also apparent that the mold can accomplish the amination of uridine with the formation of cytidine or the deamination of cytidine to uridine as well as the phosphorylations necessary for the formation of nucleic acid from the nucleosides.

The difference in the levels at which cytidine and uridine are inhibited suggests that the inhibition of at least two different reactions is involved. Evidence that the deamination of cytidine to uridine is inhibited is provided by the fact that cytidine was more readily inhibited than uridine and because the cytidine inhibition could be readily eliminated by the presence of an equimolar quantity of uridine. In the latter instance the conversion of cytidine to uridine was no longer required for nucleic acid synthesis and the inhibition of this reaction would not be expected to have the same inhibitory effect on the utilization of the two compounds for growth. At concentrations of adenosine which inhibited growth on uridine, however, it appears that it is the utilization of uridine which is affected rather than its conversion to cytidine, as this inhibition was not removed by the presence of cytidine. The nature of the reaction concerned in this case is not apparent.

The fact that the pyrimidine nucleotides, cytidylic and uridylic acids, are more strongly inhibited by adenosine than are the corresponding nucleosides is in agreement with their less efficient utilization for growth. Similarly adenosine-3-phosphate was less inhibitory than adenosine. These results are in agreement with several others which indicate that the nucleosides may play a more central rôle in nucleic acid metabolism than either the free bases or their nucleotides.

---

[4] This mutant was kindly provided by Dr. H. K. Mitchell, Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California.

The absence of adenosine inhibition in the wild type organism is in keeping with other observations that no inhibition is produced by closely related structural analogues when the substance concerned is not required for growth (11). In the wild type *Neurospora* an efficient mechanism may be present for the conversion of adenosine to adenine which later was found in these experiments not to be inhibitory. An alternative explanation may be that pyrimidine synthesis can be stimulated to balance the increased amount of adenosine present.

Of interest is the highly specific nature of the antagonism of the pyrimidine nucleosides by adenosine and guanosine and the striking reversal of the adenosine-cytidine inhibition by uridine. Unlike most inhibitions by closely related structural analogues in which high antagonist-metabolite ratios are necessary to produce inhibition, growth on cytidine was completely inhibited by an equimolar amount of adenosine. Similarly such a completely inhibitory mixture in the presence of a molecular equivalent of uridine behaved as if no antagonist at all were present. These experiments demonstrate the pronounced effect of the purine and pyrimidine nucleosides on growth in this strain of *Neurospora* and suggest a similar function in the control of growth in other organisms.

SUMMARY

The utilization of the pyrimidine ribonucleosides and ribonucleotides for growth by the pyrimidine-deficient mutant of *Neurospora* No. 1298 can be completely inhibited by the addition of adenosine or adenosine-3-phosphate to the culture medium. Adenosine is the most active antagonist, adenosine-3-phosphate is somewhat less so, and adenine has no antagonistic effect when added in comparable concentrations. The nucleotides are more readily inhibited than the nucleosides, and cytidylic acid and cytidine require less adenosine or adenosine-3-phosphate for inhibition than do uridylic acid or uridine. Guanosine and guanylic acid also inhibit the utilization of the pyrimidine compounds, but somewhat larger amounts are required. Guanine like adenine shows no inhibitory action at moderate concentrations.

The inhibition of cytidine by adenosine is strikingly reversed by the addition of an amount of uridine equal to the cytidine present. Uridine, however, is inhibited by the same concentration of adenosine regardless of whether or not an equimolar quantity of cytidine is present. These results suggest that at least two reactions may be involved in the inhibition, namely, the deamination of cytidine to uridine and the utilization of uridine itself for the synthesis of ribonucleic acid by the mold.

BIBLIOGRAPHY

1. Woolley, D. W., *J. Biol. Chem.*, **152**, 225 (1944).
2. Woods, D. D., *Biochem. J.*, **36**, 4 (1942).
3. Gladstone, G. P., *Brit. J. Exp. Path.*, **20**, 189 (1939).
4. Roblin, R. O., Jr., *Chem. Rev.*, **38**, 255 (1946).
5. Richards, M. B., *Brit. Med. J.*, **1**, 433 (1945).
6. Raska, S. B., *Science*, **105**, 126 (1947).
7. Loring, H. S., and Pierce, J. G., *J. Biol. Chem.*, **153**, 61 (1944).
8. Loring, H. S., Ordway, G. L., and Pierce, J. G., *J. Biol. Chem.*, **176**, 1123 (1948).
9. Loring, H. S., Roll, P. M., and Pierce, J. G., *J. Biol. Chem.*, **174**, 729 (1948).
10. Levene, P. A., and Bass, L. W., Nucleic Acids, American Chemical Society monograph series, New York (1931).
11. Woolley, D. W., and White, A. G. C., *J. Exp. Med.*, **78**, 285 (1943).

GROWTH AND LIPOTROPISM

II. THE EFFECTS OF DIETARY METHIONINE, CYSTINE, AND CHOLINE IN THE YOUNG WHITE RAT

By C. R. TREADWELL

(*From the Departments of Biochemistry, School of Medicine, George Washington University, Washington, and Southwestern Medical College, Dallas*)

(Received for publication, June 12, 1948)

In a previous study (1) it was shown that the total methionine requirement of rats of approximately 170 gm. initial body weight receiving a choline-free basal diet was 1200 mg. per 100 gm. of diet. This total was differentiated into a growth requirement of 600 mg. and a lipotropic requirement of 600 mg. The basal diet contained 100 mg. of cystine per 100 gm.; additional cystine did not exhibit any effect on growth or any antilipotropic activity. When the basal diet was supplemented with 100 mg. of choline per 100 gm., there was a maximum lowering of liver fat with no increase in the growth rate. Womack and Rose (2), using different experimental conditions, reported that 500 mg. of methionine and 100 mg. of cystine per 100 gm. of diet were adequate to support optimum growth in rats of an initial body weight of approximately 50 gm. Rats of this age are entering their most rapid growth period, during which it might be expected that the methionine requirement would be greater than that of 170 gm. rats which are growing at a slower rate, while our results suggested a somewhat higher growth requirement for methionine in the 170 gm. rats. In addition to the different experimental techniques, another factor which made our results difficult to correlate with those of Womack and Rose was the effect of choline, which has been shown by du Vigneaud and coworkers (3) to be present in the vitamin B complex used by Womack and Rose. While it has been reported (4, 5) that choline has a growth-stimulating effect, none was found in our study with 170 gm. rats. However, it seemed possible that in 50 gm. rats there might be a methionine-sparing effect of choline as regards growth. Thus it appeared desirable to extend our observations to rats of 50 gm. initial weight. The data presented below, when considered in conjunction with the observations in 170 gm. rats and the work of Horning and Eckstein (6) with adult male rats, indicate that under certain experimental conditions the needs of the white rat for methionine, cystine, and choline and the metabolic interrelationship of these three substances change, at least quantitatively, with the age of the animal.

EXPERIMENTAL

Young white rats weighing approximately 50 gm. (range 47 to 53 gm.) of the Sprague-Dawley strain were used. They were distributed among the dietary groups in a random manner, without regard to sex. The care of the animals has been described previously (7). The basal diet (No. 26, Tables I to III) was composed of 15.4 per cent casein, 3.2 per cent arachin, 5 per cent salt mixture (8), 2 per cent Cellu flour, 34.4 per cent glucose,[1] and 40 per cent lard. A commerical vitamin-free casein and arachin prepared from peanut flour[2] by the method of Johns and Jones (9) were used. The moisture content of the proteins was less than 1 per cent (1). The protein mixture in the basal diet provided 500 mg. of methionine and 100 mg. of cystine. The various dietary constitutents supplied a total of not more than 1 mg. of choline per 100 gm. of diet. When supplementary methionine, cystine, or choline was incorporated in the basal diet as indicated in Tables I to III, an equivalent amount of glucose was omitted. All rats received orally 0.1 cc. of U. S. P. XI cod liver oil and 0.1 cc. of a solution containing 25 γ of thiamine, 20 γ of riboflavin, 100 γ of calcium pantothenate, 100 γ of nicotinic acid, and 20 γ of pyridoxine per day. The experimental period was 21 days. The livers were removed from the animals under sodium amytal anesthesia and analyzed for total lipides (10). Apparent differences were analyzed for significance by the t method of Fisher (11), and only those showing a P value of less than 0.01 were considered significant.

Results

Table I summarizes the data obtained when Diet 26 was supplemented with increasing quantities of methionine. The response of the 50 gm. rats used in this study was markedly different from that observed in the 170 gm. rats used previously. First, some of the animals receiving the diets of lower methionine content (Diets 26, 27, 32) died between the 8th and 14th day of the experiment. Also a few of the animals which survived the experimental period showed a weight plateau lasting 3 to 5 days and a decrease in the food intake during the same interval. At autopsy, in the animals dying during the experimental period, the hemorrhagic kidney condition described by Griffith and Wade (4) was found. Also when the remaining animals were sacrificed at the end of the experimental period, gross inspection indicated in some of the animals the healed hemorrhagic kidneys described by Griffith (12). The occurrence of healed hemorrhagic kidneys was closely correlated with the occurrence of the weight plateau and the decrease in food intake. These phenomena were not observed in the older rats receiving these diets (1). Thus there was considerable variation among the young rats fed Diets 26, 27, and 32. However, in most of the animals showing a weight plateau and decrease in food intake in the period from the 8th to the 14th day, there was, following this, a slightly greater food intake and rate of growth than were observed in the animals not exhibiting these signs, so that at the end of the experimental period there was less individual variation than would have been expected. All animals surviving the experimental period were included in the averages shown in Table I.

---

[1] Generously supplied by the Corn Products Refining Company, New York.

[2] Proflo brand, kindly furnished by the Traders Oil Mill Company, Fort Worth.

The second important difference found between the young and the older rats was in the methionine requirements for growth and lipotropism. At the lower levels of methionine, no differentiation between the methionine requirements for growth and lipotropism was possible. Up to the level of 1000 mg. per 100 gm. of diet, there was a progressive increase in the growth rate and a progressive decrease in the liver lipide content. These results indicate that in the young rat both growth and lipotropism participate in the utilization of the available supply of methionine up to a level of 1000 mg. of methionine. Methionine in excess of 1000 mg. further lowered the liver fat without an effect on the growth rate; at the 1500 mg. level the liver fat content was the same as when the diet contained 500 mg. of methionine and 200 mg. of choline (Table III).

TABLE I

Methionine Requirements for Growth and Lipotropism in Young Rats Receiving Choline-Free Diets

The animals received the diets for 21 days. The average initial weights for the dietary groups ranged from 49 to 52 gm. The numbers in parentheses indicate the number of animals dying during the experimental period.

Diet No.	Methionine	Cystine	No. of rats	Food intake per day*	Gain in weight†	Liver lipides per 100 gm.	
						Moist tissue	Body weight
	mg. per 100 gm. diet	*mg. per 100 gm. diet*		*gm.*	*per cent*	*gm.*	*gm.*
26	500	100	24 (6)	5.3 ± 0.1	64.2 ± 5.5	24.1 ± 1.8	2.00 ± 0.19
27	600	100	12 (1)	5.5 ± 0.2	85.1 ± 7.7	18.3 ± 2.0	1.35 ± 0.15
32	800	100	10 (2)	5.9 ± 0.1	115.1 ± 7.1	12.0 ± 1.0	0.88 ± 0.05
33	1000	100	8	6.5 ± 0.2	140.7 ± 5.6	9.8 ± 0.4	0.61 ± 0.04
48	1300	100	18	6.3 ± 0.1	132.7 ± 4.8	8.3 ± 0.4	0.34 ± 0.01
49	1500	100	8	6.3 ± 0.2	135.0 ± 5.9	6.3 ± 0.2	0.28 ± 0.02

* Including the standard error of the mean, calculated as follows: $\sqrt{\Sigma d^2/(n-1)}/\sqrt{n}$.

† The difference between the initial and final weights expressed as per cent of initial weight.

The effect of supplementing Diet 26 with cystine is shown in Table II. The 100 mg. increase in cystine in Diet 29 produced a distinct increase in the level of liver fat. The apparent difference in the growth rate of the animals on Diets 26 and 29 is not statistically significant. Cystine at the 400 mg. level in Diet 50 produced death in 84.6 per cent of the animals between the 8th and 14th days. All of these animals died with the typical symptoms of hemorrhagic kidneys. It is apparent that the basal diet used in these experiments is well adapted for the demonstration of the effect of cystine in intensifying the development of hemorrhagic kidneys. Griffith (13) has suggested that the action of cystine in increasing the level of liver fat and intensifying the development of hemorrhagic kidneys is due to an improvement in the state of nutrition (growth); *i.e.*, when dietary cystine increases the growth rate, there is a concomitant increase in the choline and methionine (labile methyl) requirements, thereby decreasing the amount available for lipotropic action and the prevention of hemorrhagic kidneys. Both of these effects of cystine are demonstrated in this experiment without a significant increase in the growth rate. In Paper III of this series we shall present further studies which show that the suggestion of Griffith does not explain the action of cystine on liver fat and the kidneys under all experimental conditions.

TABLE II

Effect of Cystine on Growth and Lipotropism in Young Rats Receiving Choline-Free Diets

The animals received the diets for 21 days. The average initial weights for the dietary groups ranged from 49.0 to 52.2 gm. The numbers in parentheses indicate the number of animals dying during the experimental period.

Diet No.	Methionine	Cystine	No. of rats	Food intake per day*	Gain in weight†	Liver lipides per 100 gm. Moist tissue	Liver lipides per 100 gm. Body weight
	mg. per 100 gm. diet	*mg. per 100 gm. diet*		*gm.*	*per cent*	*gm.*	*gm.*
26	500	100	24 (6)	5.3 ± 0.1	64.2 ± 5.5	24.1 ± 1.8	2.00 ± 0.19
29	500	200	15 (3)	5.2 ± 0.2	69.5 ± 6.3	30.5 ± 1.1	2.73 ± 0.10
50	500	400	13 (11)	5.4	72.8	32.9	2.43

* See Table I.

† See Table I.

The data obtained when the basal diet was supplemented with choline are summarized in Table III. The symptoms of hemorrhagic kidneys were not observed in any of the animals receiving choline. The growth rate was increased by the choline supplements up to a level of 200 mg. per 100 gm. of diet, while the liver fat was decreased to an essentially normal value at the 100 mg. level. In our previous studies (1) with older animals, we have not observed a growth-promoting effect of choline. However, several other laboratories have reported this action of choline (14).

The results of the present study demonstrate some important differences in the response of 50 and 170 gm. rats to the same dietary régime. In 170 gm. rats receiving the basal diet used in these studies, 600 mg. of methionine per 100 gm. of diet are required for optimum growth, and in the absence of choline, an additional 600 mg. of methionine are needed to maintain a normal level of liver fat. In these older animals, hemorrhagic kidneys did not occur even on the unsupplemented basal diet. There is no significant lowering of liver fat by methionine in such animals until the growth requirement is satisfied. On the basis of present knowledge regarding the metabolic interrelationships of methionine and choline, it may be assumed that in 50 gm. rats receiving choline-free diets methionine is needed for growth and that labile methyl groups derived from methionine are needed for lipotropism and for the prevention of hemorrhagic kidneys. The data in Table I suggest that these three requirements compete for the available supply of methionine, but that no one takes precedence over the others. For example, if one compares the results with Diet 32 to those with Diet 26, the 300 mg. of additional methionine in Diet 32 produced an increase in the growth rate, a lowering of the liver fat, and a decrease in the incidence of hemorrhagic kidneys. However, it is apparent that none of the three requirements was completely satisfied at this level of methionine. The further increase of 200 mg. of methionine in Diet 33 produced optimum growth, a complete absence of hemorrhagic kidneys, and a nearly normal level of liver fat. When the data in Tables I and III are compared, it can be concluded that the 500 mg. of methionine and 100 mg. of cystine are not sufficient to support optimum growth, for while supplementing the basal

TABLE III

Choline Requirements for Growth and Lipotropism in Young White Rats

The animals received the diets for 21 days. The average initial weights for the dietary groups ranged from 49.0 to 52.4 gm. The numbers in parentheses indicate the number of animals dying during the experimental period.

Diet No.	Methionine	Cystine	Choline	No. of rats	Food intake per day*	Gain in weight†	Liver lipides per 100 gm.	
							Moist tissue	Body weight
	mg. per 100 gm. diet	*mg. per 100 gm. diet*	*mg. per 100 gm. diet*		*gm.*	*per cent*	*gm.*	*gm.*
26	500	100	0	24 (6)	5.3 ± 0.1	64.2 ± 5.5	24.1 ± 1.8	2.00 ± 0.19
34	500	100	100	8	6.0 ± 0.2	88.0 ± 5.1	8.2 ± 0.4	0.40 ± 0.04
35	500	100	200	8	5.7 ± 0.1	100.4 ± 5.6	6.6 ± 0.3	0.29 ± 0.17
51	500	100	300	16	6.4 ± 0.1	95.7 ± 3.0	7.2 ± 0.4	0.32 ± 0.01

* See Table I.

† See Table I.

diet with 200 mg. of choline (Diet 35) gave maximum lowering of the liver lipides, complete protection against hemorrhagic kidneys, and maximum stimulation of growth by choline, the growth rate was distinctly less than that found with the higher levels of methionine. Assuming that the choline in Diet 35 satisfied the requirements for lipotropism and prevention of hemorrhagic kidneys, then the 500 mg. of methionine which the diet contained were all available for growth. However, the 100 per cent increase in weight is less than optimum, as is shown by the group fed Diet 33; therefore the growth requirement for methionine in the 50 gm. rat is greater than 500 mg. when 100 mg. of cystine are present. Thus, the growth-stimulating effect of dietary choline in young rats, in contrast to the absence of such an effect in older animals, is most simply explained by a preferential utilization of methionine for growth in the older animals, so that the addition of choline does not decrease the demand on the available supply, whereas in the young animals, several metabolic processes are participating in the over-all utilization of the amino acid, and the introduction of choline into the diet supplies the needed methyl groups for at least two of these processes, thereby sparing methionine for growth.

SUMMARY

Young white rats (50 gm. initial weight), receiving a choline-free diet containing 18.6 gm. of protein, 500 mg. of methionine, and 100 mg. of cystine per 100 gm., grew at a rate less than optimum and developed fatty livers and hemorrhagic kidneys.

When the diet contained 1000 mg. of methionine, growth was optimum, there were no symptoms of hemorrhagic kidneys, and the level of liver fat was only slightly above normal. The total methionine requirement was between 1300 and 1500 mg. per 100 gm. of diet.

When the diet contained 200 mg. of cystine, there was a distinct antilipotropic effect without a significant increase in the growth rate. Cystine, at the 400 mg. level, produced death in 85 per cent of the animals within 14 days.

The greatest stimulation of growth by choline was observed at the 200 mg. level, which also produced a normal fat content in the liver. It is suggested that the growth-stimulating effect of choline may be attributed to a methionine-sparing action.

BIBLIOGRAPHY

1. Treadwell, C. R., *J. Biol. Chem.*, **160**, 601 (1945).
2. Womack, M., and Rose, W. C., *J. Biol. Chem.*, **141**, 375 (1941).
3. du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939).
4. Griffith, W. H., and Wade, N. J., *J. Biol. Chem.*, **131**, 567 (1939).

5. Best, C. H., Mawson, M. E. H., McHenry, E. W., and Ridout, J. H., *J. Physiol.*, **86**, 315 (1936).
6. Horning, M. G., and Eckstein, H. C., *J. Biol. Chem.*, **155**, 49 (1944).
7. Treadwell, C. R., Tidwell, H. C., and Grafa, B. G., Jr., *J. Biol. Chem.*, **149**, 209 (1943).
8. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).
9. Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, **28**, 77 (1916–17).
10. Tucker, H. F., and Eckstein, H. C., *J. Biol. Chem.*, **121**, 479 (1937).
11. Fisher, R. A., Statistical methods for research workers, Edinburgh, 7th edition (1938).
12. Griffith, W. H., *J. Nutr.*, **19**, 437 (1940).
13. Griffith, W. H., *J. Nutr.*, **21**, 291 (1941).
14. McHenry, E. W., and Patterson, J. M., *Physiol. Rev.*, **24**, 128 (1944).

GROWTH AND LIPOTROPISM

III. THE EFFECT OF SUPPLEMENTARY CYSTINE, METHIONINE, AND CHOLINE IN LOW PROTEIN DIETS

By C. R. TREADWELL

(*From the Department of Biochemistry, School of Medicine, George Washington University, Washington*)

(Received for publication, June 12, 1948)

Since the initial observation by Beeston and Channon (1) of the antilipotropic effect of cystine, there has been no entirely satisfactory explanation of this effect of the amino acid. Griffith (2) has explained the antilipotropic effect of cystine on the basis that supplementary cystine raises the metabolic level and thus creates an increased demand for lipotropic factors. This suggestion is attractive in that it does not involve a direct metabolic antagonism between cystine and choline or choline precursors such as methionine. Stetten and Grail (3) have shown that the antilipotropic effect of cystine does not involve an antagonism between cystine and choline. The data of Treadwell, Groothuis, and Eckstein (4) have been interpreted (5) as indicating a direct antagonism between cystine and methionine. However, this interpretation was not suggested by Treadwell *et al.*, and their data may be explained in other ways.

In Paper I of this series (6), it was shown that in 170 gm. rats receiving a choline-free basal diet, containing 18.6 per cent protein, 600 mg. per cent of methionine, and 100 mg. per cent of cystine, supplementary cystine up to a level of 600 mg. did not exhibit an antilipotropic effect or increase the growth rate. In a subsequent study (7), 50 gm. rats on the same basal diet showed an increase in liver fat and in the incidence of hemorrhagic kidneys without a significant increase in growth rate when given supplements of cystine.

In our studies of the various factors involved in the production and prevention of fatty livers, we have assumed that they will act either to increase or decrease the supply of methyl groups available for lipotropism. While the results obtained in this laboratory with supplementary cystine were not in disagreement with the suggestion of Griffith (2), it seemed desirable to extend our studies on cystine. It was decided to change the experimental conditions from those previously used so that the action of cystine on growth and lipotropism might be more clearly defined. In this connection it seemed especially desirable to avoid the development of the hemorrhagic kidney condition. As will be shown below, this was partly avoided by using animals of an initial weight of 100 gm. Rats of this weight, as shown by

Griffith (8), are more resistant to the condition than 40 gm. animals. Also a basal diet modeled after the one used by Osborne and Mendel (9) in their classical studies on the growth-promoting effect of cystine seemed preferable to the high protein diet used in our previous studies. For purposes of comparison, methionine and choline supplements were included in addition to cystine.

EXPERIMENTAL

White rats, of both sexes, weighing approximately 100 gm. (range 97 to 104 gm.), were used. Those used in Experiment A, Table I, were of the Carworth strain; in Experiments B, C, and D (Tables I and II), the Sprague-Dawley strain was used. The care of the animals was the same as described previously (10). Two basal diets were used. Diet 100 (Table I) consisted of 5 per cent casein, 2 per cent Cellu flour, 5 per cent salt mixture (11), 48 per cent glucose,[1] and 40 per cent lard. Diet 112 (Table II) was like Diet 100 except that it contained 9 per cent casein and 44 per cent glucose. The supplements shown in Tables I and II were introduced at the expense of the glucose. The casein was a commercial vitamin-free product. The various dietary constitutents supplied a total of less than 1 mg. of choline per 100 gm. of diet. All rats received orally 0.1 cc. of U. S. P. XI cod liver oil and 0.1 cc. of a solution containing 25 γ of thiamine, 20 γ of riboflavin, 100 γ of calcium pantothenate, 100 γ of nicotinic acid, and 20 γ of pyridoxine per day. The experimental period was 21 days. The food intake was determined daily and the weight changes were recorded three times weekly. The livers were removed from the animals under sodium amytal anesthesia and analyzed for total lipides (12). Apparent differences were evaluated by the t test of Fisher (13), and only those showing a P value of less than 0.01 were considered significant.

Results

In Experiment A (Table I), the rats receiving Diet 100 lost 8.2 per cent of their initial body weight and developed fatty livers during the 21 day period. Diet 101 contained 100 mg. of supplementary cystine. With this diet the weight loss was the same as in those receiving the basal diet. The 100 mg. of supplementary cystine exhibited a distinct antilipotropic effect, increasing the liver fat from 18.4 to 24.5 per cent. Thus, there was an antilipotropic effect of cystine in the absence of any stimulation of growth. Diet 102 contained 100 mg. of supplementary cystine and 100 mg. of choline. On this diet the animals also lost weight. However, due to the inclusion of choline, the liver fat was decreased from 24.5 to 15.1 per cent. Diet 105 contained 124 mg. of methionine, an amount of methionine equivalent to 100 mg. of cystine on the basis of sulfur. There was no stimulation of growth. Since the methionine was not used in growth, it was available for lipotropism, and there was a small decrease in the liver lipides. The 100 mg. of choline in Diet 106 lowered the liver fat to the same extent as in Diet 107. The differences in the levels of liver lipides between groups on Diets 100 and 101 and between groups on Diets 107 and 102 demonstrate the antilipotropic action of cystine. Of some interest in connection with the antilipotropic effect of cystine is the lowering of liver fat by choline in the different diets. With Diet 102 the liver fat was decreased from 24.5 to 15.1 per cent, a decrease of 9.4 per cent. With Diet 107 the lipides were decreased from 18.4 to 11.0 per cent, a lowering of 7.4 per cent. Thus, the effect of 100 mg. of choline was approximately the same in both diets. In the two diets containing cystine, the liver fat was at a higher level than in the corresponding diets without cystine. This may be interpreted as indi-

---

[1] Generously supplied by the Corn Products Refining Company, New York.

TABLE I

Effect of Supplementary Cystine, Methionine, and Choline in Rats Receiving 5 Per Cent Casein (Choline-Free) Diets

The animals received the diets for 21 days. The average initial weights for the dietary groups ranged from 99.2 to 101.1 gm. The composition of the basal diet is described in the text.

Diet No.	Supplements per 100 gm. diets			No. of rats	Food intake per day*	Change in weight†	Liver lipides per 100 gm.	
	Methionine	Cystine	Choline				Moist weight	Body weight
				Experiment A				
	mg.	mg.	mg.		gm.	per cent	gm.	gm.
100	0	0	0	8	5.3 ± 0.3	−8.2 ± 1.9	18.4 ± 0.7	1.09 ± 0.09
101	0	100	0	8	5.3 ± 0.3	−7.6 ± 1.9	24.5 ± 1.6	1.81 ± 0.21
102	0	100	100	8	5.7 ± 0.2	−3.1 ± 1.4	15.1 ± 1.2	0.77 ± 0.09
105	124	0	0	7	5.4 ± 0.2	−8.1 ± 1.9	16.3 ± 0.7	0.95 ± 0.06
106	124	0	100	6	5.4 ± 0.3	−6.8 ± 2.3	11.0 ± 0.8	0.59 ± 0.08
107	0	0	100	6	6.1 ± 0.2	−9.4 ± 1.4	11.0 ± 1.2	0.50 ± 0.06
				Experiment B				
100	0	0	0	8	4.3 ± 0.1	−18.8 ± 2.0	19.3 ± 1.3	1.10 ± 0.13
101	0	100	0	3	4.4	−21.2	27.9	1.70
108	0	300	0	8	4.6 ± 0.2	−14.6 ± 2.2	27.9 ± 1.6	2.02 ± 0.20
109	372	0	0	8	4.5 ± 0.2	−14.0 ± 1.3	12.3 ± 1.2	0.64 ± 0.10

* Including the standard error of the mean, calculated as follows: $\sqrt{\Sigma d^2/(n-1)}/\sqrt{n}$.

† The difference between the initial and final weights expressed as per cent of initial weight.

cating that in both diets the effect of the supplementary cystine was to lower the total supply of lipotropic methyl groups.

We had assumed on the basis of previous work (14, 15) that growth would be stimulated when Diet 100 was supplemented with the sulfur amino acids. It appeared that there were two possible explanations for the failure of methionine and cystine to stimulate growth on this basal diet. First, the supplements were too small to demonstrate their effect, or secondly, in addition to a low sulfur content there was some other limiting factor operating. In Experiment B (Table I), the first possibility was investigated. The supplements were increased to 300 mg. of cystine and 372 mg. of methionine. The rats used in Experiment B were from our laboratory colony which is of the Sprague-Dawley strain. These animals exhibited a greater weight loss on the basal diet than the rats of the Carworth strain used in Experiment A. The results with Diets 108 and 109 show that the failure to produce a stimulation in growth with the smaller supplements was not related to the low level of the supplements. This experiment also confirms the finding in Experiment A that the antilipotropic effect of cystine may be demonstrated in the absence of any stimulation of growth.

In Experiment C (Table II), the second possibility was investigated. The casein in the basal diet was increased to 9 per cent (Diet 112). On this diet rats from our colony lost weight slowly during the experimental period. Supplements of 100 mg. of cystine or 124 mg. of methionine produced a stimulation of growth. The growth rate was of the same magnitude with both amino acids. With Diet 113 there was an antilipotropic effect of cystine, with an accompanying stimulation of growth. The choline supplement in Diet 115 lowered the liver lipides to an essentially normal level, with no effect on the growth rate. A further stimulation of growth occurred at the higher levels of supplementary cystine and methionine. The results with cystine suggest that the young rat can use more than 100 mg. of cystine to supply its sulfur amino acid requirement. Actually the 300 mg. of cystine were more effective in stimulating growth during the first 12 to 14 days of Experiment C than the data indicate. The growth and food intake of the animals on Diet 110 closely paralleled those of the rats receiving Diet 111 up to the 12th and 14th day; then the food intake fell off and the body weight reached a plateau or decreased slightly. At autopsy there were no gross signs of hemorrhagic kidneys. These changes in food intake and growth were not observed in the other dietary groups or in the animals receiving Diet 110 in Experiment D.

The animals in Experiment D grew at a greater rate than the animals of Experiment C on comparable diets. The difference in response in the two experiments may have been due to the fact that the experiments were run at different seasons of the year or that because of unavoidable conditions the stock diet of our animal colony was changed in the interval between Experiments C and D. However, qualitatively, the results confirm those of Experiment C. Salmon (16) has recently published data, obtained with weanling rats, on the physiological relationships of protein, fat, choline, methionine, cystine, nicotinic acid, and tryptophan. Comparison of the results of the present study with those of Salmon emphasize the importance of taking into consideration the age of the experimental animals in data obtained in different laboratories. Salmon found that with weanling rats the primary deficiency in low casein diets was labile methyl groups, while in this study with 100 gm. rats, the primary deficiency was in sulfur amino acids. We have suggested previously (7) that the growth-stimulating effect of choline is most simply explained on the basis of a methionine-sparing action.

Table II

Effect of Supplementary Cystine, Methionine, and Choline in Rats Receiving 9 Per Cent Casein (Choline-Free) Diets

The animals received the diets for 21 days. The average initial weights for the dietary groups ranged from 99.1 to 101.6 gm. The composition of the basal diet is described in the text.

Diet No.	Supplements per 100 gm. diets			No. of rats	Food intake per day*	Change in weight†	Liver lipides per 100 gm.	
	Methionine	Cystine	Choline				Moist weight	Body weight
Experiment C								
	mg.	*mg.*	*mg.*		*gm.*	*per cent*	*gm.*	*gm.*
112	0	0	0	8	5.5 ± 0.3	−3.6 ± 2.7	22.2 ± 1.8	1.34 ± 0.17
113	0	100	0	8	6.1 ± 0.2	+6.4 ± 1.3	26.6 ± 1.9	1.76 ± 0.21
114	124	0	0	9	6.0 ± 0.4	+5.9 ± 2.8	19.5 ± 2.4	1.02 ± 0.15
115	0	0	100	6	5.5 ± 0.3	−2.4 ± 2.1	8.6 ± 0.5	0.36 ± 0.03
110	0	300	0	11	6.0 ± 0.2	+10.4 ± 2.9	27.9 ± 1.6	2.24 ± 0.20
111	372	0	0	9	7.0 ± 0.3	+18.4 ± 2.0	14.5 ± 2.0	0.75 ± 0.15
Experiment D								
110	0	300	0	6	7.5 ± 0.5	+28.0 ± 5.7	27.0 ± 3.5	2.03 ± 0.51
111	372	0	0	6	7.5 ± 0.2	+28.3 ± 2.0	12.9 ± 1.0	0.56 ± 0.06
115	0	0	100	6	5.8 ± 0.2	+13.1 ± 2.9	7.6 ± 0.3	0.27 ± 0.01
116	0	300	100	8	6.6 ± 0.2	+24.9 ± 2.2	12.4 ± 1.0	0.52 ± 0.07
117	372	0	100	8	7.1 ± 0.3	+31.2 ± 2.3	12.3 ± 1.1	0.45 ± 0.03

* See Table I.

† See Table I.

The failure of methionine and cystine to stimulate growth in rats receiving 5 per cent casein diets was unexpected, inasmuch as a stimulation has often been reported earlier (14, 15). A review of the earlier studies suggests that this difference may be related to the use of vitamin concentrates, while in the present study, pure vitamins were administered. The concentrates possibly supplied amino acids or other factors which are limiting in a purified 5 per cent casein diet. A second possibility is that in the present study the diets contained 40 per cent fat, so that the daily food intake was less than with diets of lower calorie content.

The results of these experiments clearly show that the antilipotropic effect of cystine is not related to an increase in the nutritional level as suggested by Griffith (2). Two other explanations of the antilipotropic effect may be cited. First, in the metabolism of cystine methyl groups may be required. Secondly, the addition of cystine may decrease the rate of removal of the methyl group from methionine by a mass action effect, for, as has been convincingly shown by du Vigneaud and coworkers (17), the demethylation of methionine yields homocysteine which combines with serine to form cystathionine which is then cleaved to liberate cystine. When cystine is added, the concentration of the end-product of the above series of reactions is increased, which may decrease the rate of the reaction.

SUMMARY

Under the conditions of the present study, supplementary cystine and methionine did not stimulate growth when added to a 5 per cent casein diet. Growth was stimulated when these amino acids were added to a 9 per cent casein diet.

The magnitude of the growth response to supplements of cystine and methionine was approximately the same for both amino acids, except during a period of decreased food intake by the cystine-fed animals in one experiment. Amounts of cystine greater than 100 mg. per 100 gm. of diet can be utilized by the rat for growth.

Cystine can produce an antilipotropic effect without an accompanying increase in the nutritional level.

Choline produces comparable decreases in the liver lipides in the presence or absence of cystine.

BIBLIOGRAPHY

1. Beeston, A. W., and Channon, H. J., *Biochem. J.*, **30**, 280 (1936).
2. Griffith, W. H., *J. Nutr.*, **21**, 291 (1941).
3. Stetten, D., Jr., and Grail, G. F., *J. Biol. Chem.*, **144**, 175 (1942).
4. Treadwell, C. R., Groothuis, M., and Eckstein, H. C., *J. Biol. Chem.*, **142**, 653 (1942).
5. McHenry, E. W., and Patterson, J. M., *Physiol. Rev.*, **24**, 128 (1944).
6. Treadwell, C. R., *J. Biol. Chem.*, **160**, 601 (1945).
7. Treadwell, C. R., *J. Biol. Chem.*, **176**, 1141 (1948).

8. Griffith, W. H., *J. Nutr.*, **19**, 437 (1940).
9. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **20**, 351 (1915).
10. Treadwell, C. R., Tidwell, H. C., and Grafa, B. G., Jr., *J. Biol. Chem.*, **149**, 209 (1943).
11. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).
12. Tucker, H. F., and Eckstein, H. C., *J. Biol. Chem.*, **121**, 479 (1937).
13. Fisher, R. A., Statistical methods for research workers, Edinburgh, 7th edition (1938).
14. Jackson, R. W., and Block, R. J., *J. Biol. Chem.*, **98**, 465 (1932).
15. du Vigneaud, V., Dyer, H. M., and Harmon, J., *J. Biol. Chem.*, **101**, 719 (1933).
16. Salmon, W. D., *J. Nutr.*, **33**, 155 (1947).
17. du Vigneaud, V., *Harvey Lectures*, **38**, 39 (1942–43).

THE VITAMIN B_6 GROUP

XIV. DISTRIBUTION OF PYRIDOXAL, PYRIDOXAMINE, AND PYRIDOXINE IN SOME NATURAL PRODUCTS*

By JESSE C. RABINOWITZ and ESMOND E. SNELL

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

(Received for publication, July 1, 1948)

Although pyridoxal, pyridoxamine, and pyridoxine are all known to occur naturally (1), almost no information concerning the distribution of these individual forms of vitamin B_6 is available. Use of three microorganisms for the individual detection of these three substances has been previously suggested (1, 2). Since *Lactobacillus casei* responds only to pyridoxal,[1] this substance could be determined directly. Both pyridoxamine and pyridoxal, but not pyridoxine, promote growth of *Streptococcus faecalis*;[1] pyridoxamine was thus obtained as the difference between the value obtained with this organism and with *Lactobacillus casei*, corrected for the difference in activity of the two compounds for the former organism. Total vitamin B_6 was determined with *Saccharomyces carlsbergensis* 4228, which responds to pyridoxal, pyridoxamine, and pyridoxine. The difference between the assay value obtained with *Saccharomyces carlsbergensis* and the sum of the values for pyridoxal and pyridoxamine was considered to represent pyridoxine.

Validity of this approach to the problem was questioned when it was found that assay of yeast and liver extracts with *Streptococcus faecalis* gave higher values for their vitamin B_6 content than did assay with *Saccharomyces carlsbergensis* (3). Subsequently this result was shown to be due chiefly to the presence in natural materials of a "bound" form of vitamin B_6, pyridoxamine phosphate, which was inactive for *Saccharomyces carlsbergensis* but active for *Streptococcus faecalis* (4). Present availability of improved hydrolytic procedure for release of vitamin B_6 from tissues (5)

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

[1] At high levels, addition of either pyridoxine or pyridoxamine to the medium permits growth of *Lactobacillus casei;* they are less than 0.001 as active as pyridoxal for this organism, however, and their contribution to the analytical values found for natural materials is entirely negligible (2). Similarly, pyridoxine is less than 0.001 as active as pyridoxamine or pyridoxal for *Streptococcus faecalis*, and its contribution to analytical values obtained with the latter organism is nil.

and of improved assay methods with *Streptococcus faecalis* (6) and *Lactobacillus casei* (7) permitted reexamination of the utility of a differential assay procedure for the various forms of vitamin B_6. Results of this study are presented below.

EXPERIMENTAL

Assay Procedures—Pyridoxal was determined with *Lactobacillus casei* (7); *Streptococcus faecalis* R was used for determination of pyridoxal plus pyridoxamine (6). *Saccharomyces carlsbergensis* 4228 was used (5, 8) for the determination of total vitamin B_6 (pyridoxal, pyridoxamine, and pyridoxine). The basal medium used in the latter procedure has been criticized (9) for the omission of nicotinic acid. In a single experiment, no difference in the apparent vitamin B_6 content of a yeast sample was noted, whether or not nicotinic acid was added to the medium. However, a review of all assays conducted on this sample showed that, when nicotinic acid had been omitted from the medium, growth was less with suboptimal levels of vitamin B_6 than when nicotinic acid was present in the medium. With heavier growth, assay values for vitamin B_6 tended to be lower. These results, summarized in Fig. 1, indicate that in some but not all assays nicotinic acid

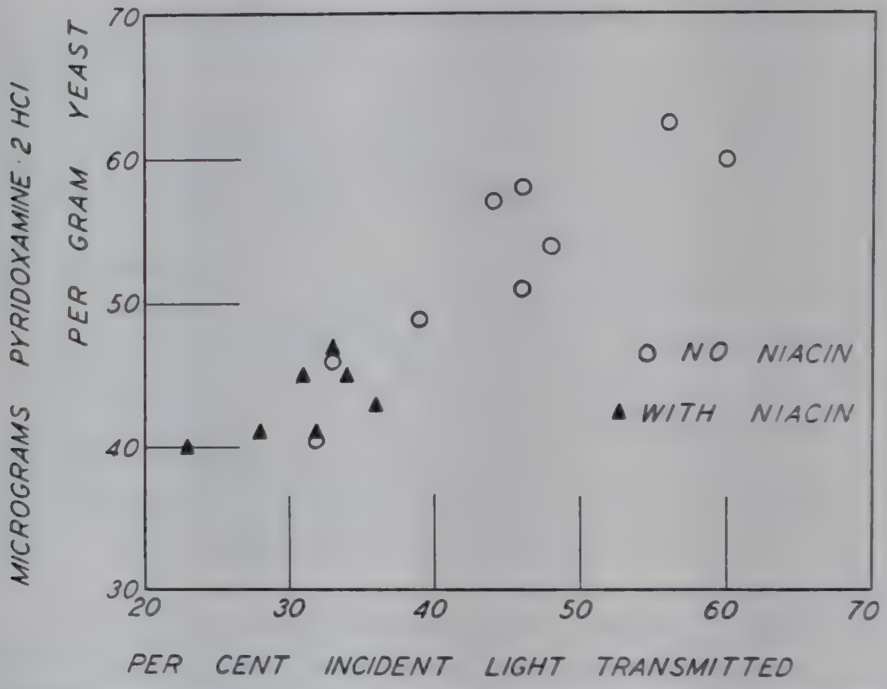


FIG. 1. The effect of nicotinic acid on the determination of vitamin B_6 with *Saccharomyces carlsbergensis*. Abscissa, per cent of incident light transmitted by a 20 hour culture of *Saccharomyces carlsbergensis* grown in the presence of 20 mγ of pyridoxamine dihydrochloride per 5 cc. Ordinate, vitamin B_6 content of a dried yeast sample calculated as micrograms of pyridoxamine dihydrochloride per gm. of yeast.

does become a growth-limiting factor in the original assay medium (8), and that under such conditions values found for vitamin B_6 may be high. For these reasons, nicotinic acid was included in the assay medium at a level of 0.5 mg. per 100 cc., as recommended by Hopkins and Pennington (9).

Whenever natural materials were assayed, an amount of sample estimated to contain 2 γ of vitamin B_6 was finely ground, placed in 180 cc. of 0.055 N hydrochloric acid, autoclaved for 5 hours at 20 pounds pressure (5), cooled, and neutralized with potassium hydroxide solution. Samples in which solid materials remained were filtered before assay.

Assay of Known Mixtures—Results of assay of mixtures of pyridoxal hydrochloride, pyridoxamine dihydrochloride, and pyridoxine hydrochloride are shown in Table I. The method for calculating the amount of each component of the mixture from the assay values requires some explanation. The dose-response curves of *Saccharomyces carlsbergensis* to pyridoxal and to pyridoxine are identical. However, the dose-response curves of both this organism and *Streptococcus faecalis* to pyridoxal and to pyridoxamine were dissimilar; *i.e.* the activity of pyridoxamine in terms of pyridoxal was not constant over the assay range used. Consequently, no constant factor for converting pyridoxal to its equivalent of pyridoxamine (or vice versa) could be used. To meet this situation, standard dose-response curves to each compound were obtained with each organism. The vitamin B_6 content of the samples was then calculated in terms of each of these standards (Columns *B* to *E*, Table I). The value found in terms of pyridoxamine divided by the value found in terms of pyridoxal thus gives a ratio which expresses the "average" activity of pyridoxal in terms of pyridoxamine over that portion of the curve used in calculating the results.

TABLE I

Determination of Pyridoxal, Pyridoxamine, and Pyridoxine in Mixtures of Known Composition

The values are expressed as millimicrograms per cc.

Sample No.	Total assay value					Amount found			Amount added		
	L. casei	*S. faecalis*		*S. carlsbergensis*							
	Standard used										
	Pyridoxal HCl (*A*)	Pyridoxal HCl (*B*)	Pyridoxamine 2HCl (*C*)	Pyridoxal HCl or pyridoxine HCl (*D*)	Pyridoxamine 2HCl (*E*)	Pyridoxal HCl (*A*)	Pyridoxamine 2HCl (*F*)	Pyridoxine HCl (*G*)	Pyridoxal HCl	Pyridoxamine 2HCl	Pyridoxine HCl
1	0.41	0.88	0.84	1.20	1.48	0.41	0.45	0.43	0.40	0.40	0.40
2	0.91	1.45	1.80	1.68	2.28	0.91	0.67	0.27	0.90	0.60	0.30

The individual components of the mixture are then calculated as follows:

$$\text{Pyridoxal hydrochloride} = A$$

$$\text{Pyridoxamine dihydrochloride} = C - \frac{C}{B} \times A = F$$

$$\text{Pyridoxine hydrochloride} = D - (A + F \times \frac{D}{E}) = G$$

where A, B, etc., are the values found with the individual assay organisms against the appropriate standard, as indicated in Tables I to IV. The validity of this method is shown by the results obtained in the analysis of the two mixtures (Table I). Recoveries of pyridoxal hydrochloride were 102 and 102 per cent, of pyridoxamine dihydrochloride 112 and 112 per cent, and of pyridoxine hydrochloride 90 and 108 per cent of the amounts added in the two samples.

The limitations of a differential assay procedure of this type should be emphasized. If one accepts ±10 per cent as the approximate limits within which values found by each of the three assay methods are reproducible (see below), then it is apparent that a variation of ±10 per cent of the total assay value found with *Streptococcus faecalis* (pyridoxal plus pyridoxamine) might be expected in the value calculated for pyridoxamine alone. Similarly, the variation encountered in the calculated value for pyridoxine will be ±10 per cent of the total assay value found with *Saccharomyces carlsbergensis*, which responds to pyridoxal, pyridoxamine, and pyridoxine. It is thus clear that values for pyridoxal are relatively accurate, those for pyridoxamine less so, and those for pyridoxine are least so. The credibility of values for pyridoxine, for example, will depend upon the proportion of the total vitamin B_6 which it represents. If this is only 10 per cent or less of the total assay value with *Saccharomyces carlsbergensis*, the value calculated for it is clearly meaningless.

A more accurate estimate of the validity of these assays may be gained from Table II. Here the results of six wholly independent analyses of a dried yeast are shown. The coefficients of error in the determination of vitamin B_6 with *Lactobacillus casei*, *Streptococcus faecalis*, and *Saccharomyces carlsbergensis* were 6.3, 8.0, and 4.2 per cent, respectively, of the mean value. The pyridoxal, pyridoxamine, and pyridoxine contents of the yeast and the probable error of each value were calculated from these average figures and their standard errors by the method previously described. From the tabulated results it is apparent that, while the values for pyridoxal and pyridoxamine are highly significant, those for pyridoxine are not, and it cannot be said with certainty whether or not any pyridoxine is present. It is certain, however, that the amount of pyridoxine present, if any, is comparatively small.

The error of the mean value, calculated on the assumption that the total assay value as determined with each organism is subject to 10 per cent error in either direction, is shown in the last line of Table II. Examination of the individual assay values shows that most, though not all of these, fall between these limits. Consequently, this procedure has been used in Tables III and IV to provide a rough measure of the probable degree of accuracy of the single assay values recorded. Although in some cases errors larger than those indicated can be expected, the satisfactory values obtained in recovery experiments (Tables I and III) show that frequently the values obtained are much more accurate than indicated.

TABLE II

Vitamin B_6 Content of Dried Yeast

The values are expressed in micrograms per gm.

Trial No.	Total assay value					Calculated content		
	L. casei	*S. faecalis*		*S. carlsbergensis*				
	Standard used							
	Pyridoxal HCl (*A*)	Pyridoxal HCl (*B*)	Pyridoxamine 2HCl (*C*)	Pyridoxal HCl or pyridoxine HCl (*D*)	Pyridoxamine 2HCl (*E*)	Pyridoxal HCl (*A*)	Pyridoxamine 2HCl (*F*)	Pyridoxine HCl (*G*)
1	4.9	42	41	34	41	4.9	36	−0.8
2	6.1	50	49	35	43	6.1	43	−6.3
3	4.1	56	53	37	45	4.1	49	−7.3
4	5.1	57	46	37	45	5.1	42	−2.9
5	6.0	42	42	38	46	6.0	36	2.2
6	4.9	54	44	40	51	4.9	40	3.7
Average ± s.e.m.*	5.2 ±0.33	50 ±4.0	46 ±2.7	38 ±1.4	45 ±1.9	5.2 ±0.33	41 ±2.9	−1.9 ±1.9
Average ±10% total assay value	5.2 ±0.5		46 ±4.6		45 ±4.5	5.2 ±0.5	41 ±5.0	−2 ±4.5

* Standard error of mean $= \sqrt{(\Sigma d_a^2)/(n(n-1))}$ where d_a is the deviation of a particular observation from the arithmetic mean and n is the number of observations (10).

Recoveries of Known Mixtures from Natural Samples—To test further the assay procedure, known mixtures of pyridoxal, pyridoxamine, and pyridoxine were added to natural materials which were then hydrolyzed as described above and assayed. Results were calculated as described earlier, and are given, together with their possible variation, in Table III. With some exceptions, recoveries are surprisingly good, even when the amounts added are small in comparison to the total assay figure. In a few cases,

TABLE III

Recovery of Mixtures of Pyridoxal, Pyridoxamine, and Pyridoxine Added to Natural Products

Sample	Total assay value					Content			Amount recovered		
	L. casei	*S. faecalis*		*S. carlsbergensis*							
	Standard used										
	Pyridoxal HCl (*A*)	Pyridoxal HCl (*B*)	Pyridoxamine 2HCl (*C*)	Pyridoxal HCl or pyridoxine HCl (*D*)	Pyridoxamine 2HCl (*E*)	Pyridoxal HCl (*A*)	Pyridoxamine 2HCl (*F*)	Pyridoxine HCl (*G*)	Pyridoxal HCl	Pyridoxamine 2HCl	Pyridoxine HCl
	γ per gm.	*γ per gm.*	*γ per gm.*	*γ per gm.*	*γ per gm.*	*γ per gm.*	*γ per gm.*	*γ per gm.*	*γ per gm.*	*γ per gm.*	*γ per gm.*
Wilson 1:20 liver powder	9.8	46	30	48	58	9.8 ±1.0	24 ±5	18 ±6			
Wilson 1:20 liver powder + added vitamin B₆	19	148	102	221	268	19 ±2	89 ±15	128 ±27	9 ±2	65 ±15	110 ±27
Added vitamin B₆									10	60	100
Cerogras	2.7	7.3	6.8	12	16	2.7 ±0.3	4.3 ±0.7	6 ±2			
" + added vitamin B₆	8.1	26	21	54	67	8.1 ±0.8	14 ±3	35 ±7	5.4 ±0.8	10 ±3	29 ±7
Added vitamin B₆									5	10	25
Dried Yeast I	5.1	57	46	37	45	5.1 ±1	42 ±6	−3 ±5			
" " I + added vitamin B₆	18	90	69	74	88	18 ±2	55 ±9	10 ±9	13 ±2	13 ±9	10 ±9
Added vitamin B₆									12	12	12
Dried Yeast II	4.9	54	44	40	51	4.9 ±0.5	40 ±5	4 ±5			
" " II + added vitamin B₆	9.7	144	121	216	278	9.7 ±1.0	113 ±14	118 ±28	4.6 ±1.0	73 ±14	114 ±28
Added vitamin B₆									5	50	100
	mγ per cc.	*mγ per cc.*	*mγ per cc.*	*mγ per cc.*	*mγ per cc.*	*mγ per cc.*	*mγ per cc.*	*mγ per cc.*	*mγ per cc.*	*mγ per cc.*	*mγ per cc.*
Urine	100	293	258	190	242	100 ±10	170 ±29	−43 ±24			
" + added vitamin B₆	199	678	562	772	953	199 ±20	397 ±68	253 ±95	99 ±20	227 ±68	253 ±95
Added vitamin B₆									100	200	400

e.g. pyridoxamine recovery from Dried Yeast II, recoveries were less accurate than were expected.

TABLE IV

Pyridoxal, Pyridoxamine, and Pyridoxine Content of Some Natural Materials

Sample	Pyridoxal HCl	Pyridoxamine 2HCl	Pyridoxine HCl
	γ per gm.	*γ per gm.*	*γ per gm.*
Chick liver	38 ± 4	46 ± 8	0 ± 10
" heart	8.0 ± 0.8	12 ± 2	−1 ± 2
" breast muscle	37 ± 4	1 ± 4	8 ± 6
" brain	11 ± 1	7 ± 2	3 ± 3
" kidney	30 ± 3	26 ± 6	−3 ± 6
Rat liver	29 ± 3	9 ± 4	9 ± 5
" heart	16 ± 2	9 ± 3	1 ± 3
" breast muscle	22 ± 2	−1 ± 2	5 ± 3
" brain	9.2 ± 0.9	4 ± 3	4 ± 2
" kidney	33 ± 3	13 ± 5	10 ± 6
" spleen	3.8 ± 0.4	0.7 ± 0.4	−0.4 ± 0.5
" sarcoma	2.3 ± 0.2	9 ± 1	3 ± 2
Fresh beef liver	7.0 ± 0.7	31 ± 4	−3 ± 4
Wilson 1:20 liver powder	6.1 ± 0.6	31 ± 4	15 ± 5
Liver concentrate, Sharp and Dohme	16 ± 2	47 ± 8	6 ± 15
Frozen fish	10 ± 1	22 ± 3	−1 ± 3
Dried yeast, brewers'	4.9 ± 0.5	36 ± 4	−1 ± 4
Fresh " bakers'	7.5 ± 0.8	11 ± 2	0 ± 2
Dried *Penicillium* mycelium	3.5 ± 0.4	9 ± 1	4 ± 2
Fresh whole milk, *γ per cc.*	0.32 ± 0.03	0.09 ± 0.09	−0.02 ± 0.05
" egg white	0.19 ± 0.02	0.14 ± 0.03	0.39 ± 0.08
" " yolk	11 ± 1	4 ± 2	0 ± 2
" whole egg	5.6 ± 0.6	1.2 ± 0.7	0 ± 0.9
Cerogras	4.1 ± 0.4	2.5 ± 0.7	4.0 ± 1.0
Fresh celery	7.8 ± 0.8	2.5 ± 1.1	9 ± 2
" leaf lettuce	12 ± 1	−1 ± 2	9 ± 2
" green pepper	8.2 ± 0.8	53 ± 6	20 ± 8
Whole lemon	3.0 ± 0.3	0.7 ± 0.4	10 ± 2
Fresh carrot	2.1 ± 0.2	0.3 ± 0.3	7 ± 1
Whole wheat	1.9 ± 0.2	3.7 ± 0.4	9 ± 2
Wheat germ	0.87 ± 0.09	0.7 ± 0.2	3.1 ± 0.7
Split peas	0.47 ± 0.05	0.43 ± 0.1	1.3 ± 0.3
Yellow corn-meal	1.4 ± 0.1	1.1 ± 0.2	0 ± 0.3
Vitab	1.9 ± 0.2	10 ± 2	79 ± 11

When the method was applied to human urine, a negative value for pyridoxine was found whose magnitude was considerably greater than the expected error, even if it were assumed that no pyridoxine was present. In this case, recoveries of added pyridoxal and pyridoxamine were within experimental error, while recovery of pyridoxine was low. These results indicate the presence in human urine of materials, toxic for *Saccharomyces carlsbergensis*, which suppress the growth response of this organism to vitamin B_6, but which do not affect the responses of *Streptococcus faecalis* or *Lactobacillus casei*.

Distribution of Pyridoxal, Pyridoxamine, and Pyridoxine in Natural Materials—Results of assay of a variety of tissues and other natural materials are given in Table IV. The vegetables, fish, meat, and dairy products were purchased in a local grocery. Chick and rat tissues were from experimental animals on stock rations, and were prepared for analysis immediately following death from decapitation or asphyxiation. Tissues from four to six animals were pooled for assay. After homogenization in the Waring blendor, separate samples were taken for hydrolysis and for dry weight determinations. Values are expressed in terms of micrograms per gm. of dry material.

Over 80 per cent of the vitamin B_6 in all fresh animal tissues studied was present as pyridoxal and pyridoxamine. In most of these samples, pyridoxal was the predominant form. In most cases, it was not possible to demonstrate the presence of any pyridoxine; if present at all, it constitutes only a minor fraction of the total vitamin B_6 of these tissues. With the exception of the fresh rat liver, pyridoxamine was the predominant form of the vitamin in all liver samples analyzed. Pyridoxal and pyridoxamine were also the predominant forms of vitamin B_6 in frozen fish, fresh and dried yeast, *Penicillium* mycelium, milk, and eggs. Although pyridoxal and pyridoxamine were also prominent in the plant products tested, it was evident that pyridoxine was also present to a very significant extent. In this respect, the contrast between plant and animal products is quite clear. Vitab, an extract of rice bran, is very high in pyridoxine and low in pyridoxal and pyridoxamine. It is significant, therefore, that the only procedures for isolation of pyridoxine which have been described in detail utilized rice bran as a starting material.

DISCUSSION

Aside from microbiological procedures, only two methods, both physicochemical, have been suggested for determination of pyridoxal, pyridoxamine, and pyridoxine. Neither of these has been adapted to assay of natural materials.

The reaction of pyridoxine with diazotized sulfanilic acid was first considered as an analytical method for the determination of pyridoxine by Kuhn and Low (11). Ormsby, Fisher, and Schlenk (12) have shown that pyridoxal, pyridoxamine, and pyridoxine form derivatives with this reagent of differing colors, which can be differentiated spectroscopically. This reaction was used to demonstrate presence of both pyridoxal and pyridoxamine in a purified preparation of transaminase. Hopkins and Pennington (9), however, noted that the colors produced with this reagent were transitory and lacked specificity.

A method for determining pyridoxine in the presence of pyridoxal and pyridoxamine has been developed by Melnick *et al.* (3). This method is based on a reaction, first noted by Stiller, Keresztesy, and Stevens (13), between pyridoxine and 2,6-dichloroquinone chloroimide, later modified by Scudi, Bastedo, and Webb (14). The reaction does not offer a means of differentiating pyridoxal from pyridoxamine, however, and its specificity when applied to natural materials is not known.

Although satisfactory in principle, the method described in this paper has severe limitations which should be recognized. Since pyridoxamine and pyridoxine are not determined directly, but by difference, the error involved in their determination is largely dependent on the relative proportion of these substances to the total vitamin B_6 of the sample. With the present method, it is not possible to demonstrate with certainty the presence of pyridoxine in a sample when less than 10 per cent of the total vitamin B_6 is pyridoxine, and errors are large even when more than this is present. This was the case with all animal tissues investigated. Likewise the present method does not allow the determination of pyridoxamine in a sample in which the pyridoxamine constitutes less than 10 per cent of the sum of the pyridoxal and pyridoxamine of the sample. Since pyridoxal is determined directly, its determination is quite satisfactory.[2] Unfortunately, no microorganisms are known which respond specifically to pyridoxamine or pyridoxine, so that their direct determination is not possible. A direct determination of pyridoxamine could be obtained, however, by quantitatively destroying pyridoxal with acetone and alkali (1) and assaying the resulting mixture for pyridoxamine with *Streptococcus faecalis*. Assay of the same mixture with yeast would then provide a differential determination of pyridoxine involving only two instead of three microorganisms, which should be somewhat more accurate than the method used here. This refinement of the present procedure has not been generally applied as yet. For reliable microbiological estimates of the small amounts of pyridoxine present in animal and some other tissues, however, some procedure for its separation from pyridoxal and pyridoxamine, such as that recently suggested by Winsten and Eigen (16), will be required, so that it can be estimated separately.

---

[2] The loose condensation products (Schiff's bases?), which pyridoxal forms with amino acids and other amines, and the thiazolidine derivative formed with cysteine (15, 16) may occur naturally. These products readily decompose to form pyridoxal, and like pyridoxal are fully active for *Lactobacillus casei* (15). It has been emphasized (16) that the transamination reaction between pyridoxal and amino acids, previously shown to occur when solutions containing these substances are heated (2, 17), occurs also to some extent in the cold, and that figures for the pyridoxal content of natural materials may hence be of doubtful significance. Previous work has shown (7), however, that this reaction does not occur under the hydrolytic conditions employed in this investigation. It is also minimized or completely avoided even at neutrality by use of dilute solutions. The analytical values given for fresh tissues, therefore, should reflect accurately the amount of pyridoxal actually present.

The present findings confirm and expand previous indications (1, 6) that pyridoxine, if present at all, constitutes quantitatively a very minor portion of the vitamin B_6 of animal tissues and many other products. Despite this fact, and the fact that by no previously applied method of analysis can the various forms of this vitamin be distinguished, numerous workers continue to refer misleadingly and erroneously to the "pyridoxine" content of natural materials. If by *pyridoxine* is meant the specific chemical compound to which this name was given (18), it is evident that the pyridoxine content of such materials is unknown and is not determined by the methods used. If, on the other hand, *pyridoxine* is used as a group name to include all substances curative of vitamin B_6 deficiency in animals (including pyridoxal and pyridoxamine (2, 19, 20)), then it obviously should not be used also as the name for the specific compound, 2-methyl-3-hydroxy-4,5-bis-(hydroxymethyl)pyridine.

The present confusion in nomenclature results from retention of the name "pyridoxine" as synonymous with "vitamin B_6" and at the same time as a name for a specific compound. A more rational and less confusing notation would result if the term "pyridoxine" were reserved only for the specific compound and the term "vitamin B_6" were used as a group name to include all three compounds.

SUMMARY

A method for the differential determination of pyridoxal, pyridoxamine, and pyridoxine in natural products is described. This method takes advantage of differences in the specificity of response of *Lactobacillus casei*, *Streptococcus faecalis*, and *Saccharomyces carlsbergensis* to the different forms of vitamin B_6. Although the principle of this method was suggested previously (2), its application to the analysis of natural materials was possible only after further development of the three individual assay procedures involved and a clarification of the hydrolytic procedures used in the liberation of the vitamins from natural materials, where they occur largely in more complex forms unavailable to the microorganisms.

Analysis of known mixtures of the three compounds by the method described gave satisfactory results. With few exceptions, satisfactory recoveries of the vitamins from natural materials were obtained. A significant

exception occurred in the case of the recovery from urine, in which the amount of pyridoxine recovered was lower than could be accounted for by analytical errors. Limitations common to differential methods of this type are discussed. It is shown that the accuracy of the figures for pyridoxine depends upon the relative proportion of this substance to pyridoxal and pyridoxamine. When pyridoxine comprises less than 10 per cent of the total vitamin B_6, it cannot be determined by this procedure.

Analytical values for the pyridoxal, pyridoxamine, and pyridoxine content of natural materials are given. Pyridoxal and pyridoxamine were the predominant forms of vitamin B_6 in hydrolyzed animal tissues and yeasts, with only slight indications or none at all of the presence of pyridoxine in these samples. Pyridoxine was more evident in plant materials, in which it occurred in amounts as large or larger than those of pyridoxal and pyridoxamine.

BIBLIOGRAPHY

1. Snell, E. E., *J. Biol. Chem.*, **157**, 491 (1945).
2. Snell, E. E., and Rannefeld, A. N., *J. Biol. Chem.*, **157**, 475 (1945).
3. Melnick, D., Hochberg, M., Himes, H. W., and Oser, B. L., *J. Biol. Chem.*, **160**, 1 (1945).
4. Rabinowitz, J. C., and Snell, E. E., *J. Biol. Chem.*, **169**, 643 (1947).
5. Rabinowitz, J. C., and Snell, E. E., *Anal. Chem.*, **19**, 277 (1947).
6. Rabinowitz, J. C., and Snell, E. E., *J. Biol. Chem.*, **169**, 631 (1947).
7. Rabinowitz, J. C., Mondy, N. I., and Snell, E. E., *J. Biol. Chem.*, **175**, 147 (1948).
8. Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. M., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 141 (1943).
9. Hopkins, R. H., and Pennington, R. J., *Biochem. J.*, **41**, 110 (1947).
10. Crumpler, T. B., and Yoe, J. H., Chemical computations and errors, New York (1940).
11. Kuhn, R., and Low, I., *Ber. chem. Ges.*, **72**, 1453 (1939).
12. Ormsby, A. A., Fisher, A., and Schlenk, F., *Arch. Biochem.*, **12**, 79 (1947).
13. Stiller, E. T., Keresztesy, J. C., and Stevens, J. R., *J. Am. Chem. Soc.*, **61**, 1237 (1939).
14. Scudi, J. V., Bastedo, W. A., and Webb, T. J., *J. Biol. Chem.*, **136**, 399 (1940).
15. Snell, E. E., and Rabinowitz, J. C., *J. Am. Chem. Soc.*, **70**, 3432 (1948).
16. Winsten, W. A., and Eigen, E., *Proc. Soc. Exp. Biol. and Med.*, **67**, 513 (1948).
17. Snell, E. E., *J. Am. Chem. Soc.*, **67**, 194 (1945).
18. György, P., and Eckardt, R. E., *Nature*, **144**, 512 (1939).
19. Sarma, P. S., Snell, E. E., and Elvehjem, C. A., *J. Biol. Chem.*, **165**, 55 (1946).
20. Sarma, P. S., Snell, E. E., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **63**, 284 (1946).

A MICROBIOLOGICAL METHOD FOR THE DETERMINATION OF ADENINE*

BY JAY V. BECK

(From the Department of Bacteriology, Pennsylvania State College, State College)

(Received for publication, July 19, 1948)

In spite of marked advances and high interest in purine and nucleic acid biochemistry in recent years (7), adequate methods in quantitative purine chemistry are still not available. At present there is no method suitable for the accurate estimation of adenine in mixtures of purines; a similar situation exists for the other common purines, with the exception of uric acid.[1] The newly proposed chromatographic (9) and counter-current distribution (8) procedures, as well as the method of differential spectrophotometry (5), may prove valuable for the determination of purines in mixtures, but their usefulness has not as yet been demonstrated. The proposed use of a mutant strain of *Neurospora crassa* has been shown to have little value for the estimation of purines in mixtures (6). Thus, the report that *Clostridium acidiurici* is able to bring about a fairly complete decomposition of guanine, xanthine, and hypoxanthine, but not of adenine (1), led to the suggestion that this highly specific bacterium may be useful for the quantitative estimation of this purine.

This paper reports progress that has been made in developing a specific microbiological method for the estimation of adenine. It is shown that highly accurate adenine determinations in purine mixtures are possible by the use of *Clostridium acidiurici*. The purine content of a commercial sample of yeast nucleic acid (ribonucleic acid) is also reported.

Methods and Materials

Heavy cell suspensions of *Clostridium acidiurici* (strain 9a) were prepared as previously described (1) with the exception that a medium containing 0.2 per cent uric acid and 0.02 per cent hypoxanthine was used for growth of the organisms. Cells grown on this medium have a higher activity towards hypoxanthine than those grown on uric acid alone. Substrate decompositions were carried out in small evacuated reaction vessels or

---

* Authorized for publication on July 12, 1948, as paper No. 1452 in the Journal Series of the Pennsylvania Agricultural Experiment Station. This work was supported in part by a research grant from the National Cancer Institute of the National Institute of Health, United States Public Health Service.

[1] Since adequate methods for uric acid are available and also because it is readily removed from biological systems by action of uricase, it has not been considered in this paper.

Thunberg tubes. The total volume of cells plus substrate ranged from 4 to 6 ml. Blank determinations on the substrate and cell suspension incubated separately were carried out and the indicated corrections were made to the analytical data.

Ammonia, acetic acid, and carbon dixoide were determined as previously outlined. Acetic acid was identified by Duclaux distillation.

Yeast nucleic acid (YNA) was hydrolyzed by the method of Graff and Maculla (2). It was found that complete liberation without decomposition of the purines was accomplished by heating at 100° with about 1 N HCl over a rather wide period of time (45 minutes to 2 hours). 1 gm. of YNA was dissolved in 10 ml. of 1 N HCl, heated to boiling for 1 hour, cooled, neutralized, and diluted to a volume of 100 ml. Aliquot portions of this solution were taken for further work.

Free purines were isolated by the method of Hitchings and Fiske (4). This method is fast, and quantitative recovery (98 to 100 per cent) of free purines, whether present singly or in mixtures, is regularly attained.

The purines used were obtained from commercial sources and were found to contain 98 to 99 per cent of the theoretical N content. The yeast nucleic acid, also a commercial sample, had a nitrogen content of only 92 per cent of the theoretical value, based on the polytetranucleotide formula. No attempt was made to purify this material further.

EXPERIMENTAL

Clostridium acidiurici (strain 9a) was shown (1) to be able to carry out a fairly complete decomposition of xanthine, guanine, and hypoxanthine, but to have little or no action on adenine. Work on these purine decompositions has yielded data which are summarized in Table I. Ammonia, carbon dixoide, and acetic acid are shown to account for 91 to 97 per cent of the carbon and nitrogen of the purines decomposed. The values shown are averages of a number of determinations made over a period of several years. The range of values is given to indicate the variation which may be expected in these determinations. The experimental error in the ammonia determinations as carried out in this work is seen to be about ±2 per cent, for carbon dixoide about ±4 per cent, and somewhat larger, ±8 to 9 per cent, for acetic acid. The conversion of purine N to NH_3-N is seen to vary from 93 to 97 per cent, with an average of 95 per cent.

Ammonia production from adenine has not been detected[2] in these experiments. There is some volatile acid produced from adenine; although no attempt has been made to identify this acid, it may be expected to consist of acetic acid.

---

[2] Reported NH_3 production from adenine (1) has not been found in these more recent experiments.

Since these experiments show a nearly complete and constant conversion of xanthine, guanine, or hypoxanthine nitrogen to NH_3-N, while no such reaction occurs with adenine, ammonia production by *Clostridium acidiurici* from mixtures of purines may be expected only from the former three purines. A number of experiments have been carried on which demonstrate this to be the case.

TABLE I

Products of Action of Cell Suspensions of Clostridium acidiurici on Purines

The average values shown are from five or more analyses.

Substrate*	Production per 100 moles substrate			Per cent recovery	
	Acetic acid	Carbon dioxide	Ammonia	Nitrogen	Carbon
	moles	*moles*	*moles*		
Xanthine, average	89	279	380	95	91
" range	88–96	276–286	368–384	92–96	90–96
Guanine, average	84	286	485	97	91
" range	79–89	279–303	468–490	95–98	87–96
Hypoxanthine, average	116	225	371	93	91
" range	111–120	220–228	360–376	90–94	88–94
Adenine, average	9†	0	0		

* Quantities of substrate varied from 0.5 to 3 mg. of purine N.

† Not identified as acetic acid.

TABLE II

Determination of Adenine Content of Mixtures of Purines

4 ml. of substrate, 1 ml. of cell suspension, 1 ml. of phosphate buffer, pH 7.2. Incubated 14 hours at 35° in evacuated Thunberg tube.

Tube No.	Purine nitrogen taken				Ammonia N found*	Adenine N, by difference	Adenine recovered
	Xanthine	Guanine	Hypoxanthine	Adenine			
	mg.	*mg.*	*mg.*	*mg.*	.	*mg.*	*per cent*
1	0.439	0.521	0.395	0.563	1.356	0.562	100
2	0.439	0.521	0.395	0.563	1.345	0.573	102
3	0.439	0.521	0.395	0	1.358	−0.003	
4	0.439	0	0.395	0.563	0.821	0.576	102
5	0	0.521	0.395	0.563	0.914	0.555	98
6	0.439	0.521	0.790	0	1.760	−0.010	

* Corrected for average of 95 per cent recovery from purines.

The results of one such experiment are summarized in Table II. A cell suspension of *Clostridium acidiurici* was allowed to act on a solution containing the indicated mixture of purines at pH 7.2 for 14 hours at 35°.

Ammonia production was then determined. The ammonia production shown is a corrected value obtained by dividing the actual ammonia found by the factor 0.95, which is the average value for conversion of purine N shown in Table I. This introduces some slight error in those cases in which the decomposable purines are not present in approximately equal amounts. Adenine N is then calculated by subtracting the corrected NH_3-N from the total purine N, which is known in this experiment, or which may be determined experimentally as will be shown later. The data show that the adenine content of the mixtures as calculated from analytical values agrees satisfactorily with the known amount of adenine taken. The percentage recovery in the data presented here is 98 to 102 per cent, which is about the experimental error for the ammonia determination shown in Table I. Other data indicate that an accuracy of ±3 per cent may be expected.

TABLE III

Analysis of Purine Content of Yeast Nucleic Acid

The values are per cent of yeast nucleic acid.

	Total N	Purine N	Guanine N (non-adenine purine N)		Adenine N, by difference
			Purines not isolated	Purines isolated	
Theoretical values	16.55	10.06*	5.03*	5.03*	5.03*
Determined "	15.09	9.94	5.06	4.95	4.99

* Based on yeast nucleic acid N content of 15.09 per cent.

It is obvious that application of this procedure to complex biological material depends on a separation of the purines from other nitrogenous substances in order to arrive at a true value of the purine N. A separation is necessary also because it has been shown that glycine and perhaps other nitrogenous compounds are decomposed by *Clostridium acidiurici* with ammonia production in the presence of purines (1). The method of Hitchings and Fiske has been found to be a satisfactory means of separating free purines and of producing a solution of the purines suitable for decomposition by cell suspensions of *Clostridium acidiurici*.

The data presented in Table III illustrate the use of cell suspensions of *Clostridium acidiurici* in the determination of the purine content of yeast nucleic acid. Total N was determined on an aliquot of the unhydrolyzed sample. Free purines were isolated from the hydrolyzed sample, and subjected to total N determination and to action of cell suspensions of *Clostridium acidiurici*. The hydrolyzed sample was also treated with cell suspensions before isolation of the free purines. Ammonia N was determined on the samples subjected to action of the bacterial cells.

The results show that the purine N is very nearly that expected from consideration of the total N content of 15.09 per cent and the theoretical polytetranucleotide formula for yeast nucleic (ribonucleic) acid. The total purine N determined on the isolated purine fraction is found to be 9.94 per cent, as compared with a theoretical value of 10.06 per cent. The adenine N content measured by the non-decomposable purine N is shown to be 4.99 per cent as compared with a theoretical value of 5.03 per cent. The decomposable purine N, presumably guanine as will be shown later, is 4.95 per cent, theoretical value 5.03 per cent. A similar value for guanine N in the hydrolyzed but not separated purine fraction is seen to be 5.06 per cent. The series of values reported in Table III are from a single analysis. However, almost identical values have been obtained in three additional complete analyses as well as several other partially complete analyses.

TABLE IV

Identification of Lecomposable Portion of Yeast Nucleic Acid

The values are averages of five or more analyses; the range of values is shown in parentheses.

Substrate	Carbon dioxide produced*	Acetic acid produced*
Hydrolyzed YNA, purines not isolated	57 (53–62)	19 (18–21)
Purines isolated from YNA	56 (55–57)	16 (15–17)
Guanine†	59	17
Xanthine†	73	23
Hypoxanthine†	61	31

* Based on NH_3 production as 100.

† Calculated from average values shown in Table I.

Identification of the decomposable purine fraction is made possible by consideration of the quantities of acetic acid and carbon dioxide formed during the dissimilation process. The values for acetic acid and carbon dioxide production from known samples of the three labile purines are shown in Table IV calculated to the basis of ammonia production equal to 100 from the data in Table I. These values may be compared with similar values found on analysis of fermented purines from yeast nucleic acid. The values for the decomposable portion of the purines of yeast nucleic acid are slightly lower than the values for pure guanine, but are widely different from the values for xanthine or for hypoxanthine.

Some preliminary work has been done to determine the effect on analytical results of additions of purines to YNA before hydrolysis. Quantitative recoveries of such added purines in the purine N fractions have been achieved. Indications are that quantities of added purine N which are less than 5 per cent of the total purine N in the sample will not affect the acetic acid and carbon dioxide values enough to be detected. Presence of added hypoxanthine is most easily detected due to the greater relative acid production, as seen in Table IV.

DISCUSSION

The specificity of *Clostridium acidiurici* makes possible a quantitative separation of adenine from other commonly occurring purines. A method for the estimation of adenine has been developed in which the non-decomposable purine N is assumed to be adenine N. This assumption is valid in most instances because most biological systems contain only common purines (uric acid, xanthine, guanine, hypoxanthine, and adenine). However, in some cases, large amounts of purines or purine derivatives are present which are precipitable as cuprous complexes and which are non-decomposable by *Clostridium acidiurici*. Therefore, general specificity of the method for adenine cannot be claimed. In spite of this, it is believed that the method has value in purine chemistry. Further work is being done on chemical and microbiological means for the identification of adenine in the non-decomposable fraction of purine mixtures.

Identification of decomposable purines when present singly or in binary mixtures with adenine is made possible by determination of the ratios of ammonia, carbon dioxide, and acetic acid produced. The ammonia production is a measure of the quantity of such purine decomposition. Attempts have been made to estimate amounts and identities of three decomposable purines in mixtures by use of simultaneous equations. But the magnitude of experimental errors shown in Table I is too large for such a method to be successfully employed. The existence of mixtures of decomposable purines can be detected if appreciable quantities of all components are present, but the identification of the individual purines is not yet possible.

Gulland (3) has found the ratio of purine N to pyrimidine N in ribonucleic acid to be 1.86 instead of 2.00 which is predicted by the tetranucleotide formula. From the data given in Table III the purine N to pyrimidine N[3] ratio of the yeast nucleic acid studied may be calculated to be 1.93. However, since any loss of purine N increases the value for pyrimidine N, it is believed that the difference in ratio between 1.93 and 2.00 is well within the experimental error for this determination. The quantities of guanine N and non-decomposable purine N (presumably adenine) are also equal within experimental error. The data thus support the polytetranucleotide formula for yeast nucleic (ribonucleic) acid.

---

[3] Pyrimidine N calculated as the difference between total N and purine N.

SUMMARY

1. A microbiological method for the estimation of adenine in the presence of other commonly occurring purines is presented. The method is based on the specificity of *Clostridium acidiurici.*

2. Determination and identification of other common purines in binary mixtures with adenine is possible by this procedure.

3. The purine content of a commercial sample of yeast nucleic acid is found to agree very well with the polytetranucleotide formula.

BIBLIOGRAPHY

1. Barker, H. A., and Beck, J. V., *J. Biol. Chem.*, **141,** 3 (1941).
2. Graff, S., and Maculla, A., *J. Biol. Chem.*, **110,** 71 (1935).
3. Gulland, J. M., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **12,** 95 (1947).
4. Hitchings, G. H., and Fiske, C. H., *J. Biol. Chem.*, **140,** 491 (1941).
5. Kalckar, H. M., *J. Biol. Chem.*, **167,** 429 (1947).
6. Loring, H. S., and Fairley, J. L., Jr., *J. Biol. Chem.*, **172,** 843 (1948).
7. Nucleic acids and nucleoproteins, Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **12,** (1947).
8. Tinker, J. F., and Brown, G. B., *J. Biol. Chem.*, **173,** 585 (1948).
9. Vischer, E., and Chargaff, E., *J. Biol. Chem.*, **168,** 781 (1947).

IMMUNOLOGICAL AND ELECTROPHORETIC STUDIES ON HUMAN γ-GLOBULINS*

By B. V. JAGER, EMIL L. SMITH, MARGARET NICKERSON, and DOUGLAS M. BROWN

(From the Department of Medicine, and the Laboratory for the Study of Hereditary and Metabolic Disorders, University of Utah School of Medicine, Salt Lake City)

(Received for publication, June 29, 1948)

Apart from physical and chemical data, there is immunological evidence for the presence of several globulins in animal and human sera (1–3). Kendall (3) isolated from human serum, by salt precipitation, a globulin fraction which satisfied the immunological criteria for a homogeneous protein. Other globulin fractions could not be shown to be immunologically homogeneous, although they differed antigenically from the homogeneous fraction which is now known to be γ-globulin (4).

Determination of the protein content of the precipitate produced by addition of varying amounts of *homogeneous* antigen to a constant quantity of homologous antiserum permits construction of a curve from which the antigen present in an unknown solution may be estimated from the protein content of the antigen-antibody precipitate (5). With an antiserum specific to γ-globulin, Kendall (3) and Kabat, Glusman, and Knaub (6) have determined the amount of reactive antigen present in normal and abnormal human serum and other body fluids.

We have examined highly purified γ-globulin fractions II-1,2 and II-3 as to immunological homogeneity and immunological equivalence. The quantity of reactive antigen precipitated by the γ-globulin antiserum was measured in normal and abnormal human sera. The values obtained with this immunological technique have been compared with electrophoretic estimations of γ-globulin in the same sera. Some electrophoretic and ultracentrifugal observations on human γ-globulins are also presented.

Electrophoretic and Ultracentrifugal Observations

The γ-globulins used in this study were prepared by Method 9 of Oncley *et al.* (7) and are the same lyophilized preparations which have been previously analyzed for carbohydrate and various amino acids (8). These preparations are II-1,2, Squibb 324-329, and II-3, Squibb 341.

The proteins were studied electrophoretically in a Tiselius apparatus

---

* This investigation was supported by grants from the Life Insurance Medical Research Fund and the United States Public Health Service.

equipped with the Longsworth schlieren scanning device with univalent buffers at an ionic strength of 0.1. Most of the observations were made at a protein concentration of about 1 per cent except in the region of the isoelectric point of the II-3 globulin, where its insolubility necessitated the use of lower concentrations. Some of the patterns which were obtained are shown in Fig. 1.

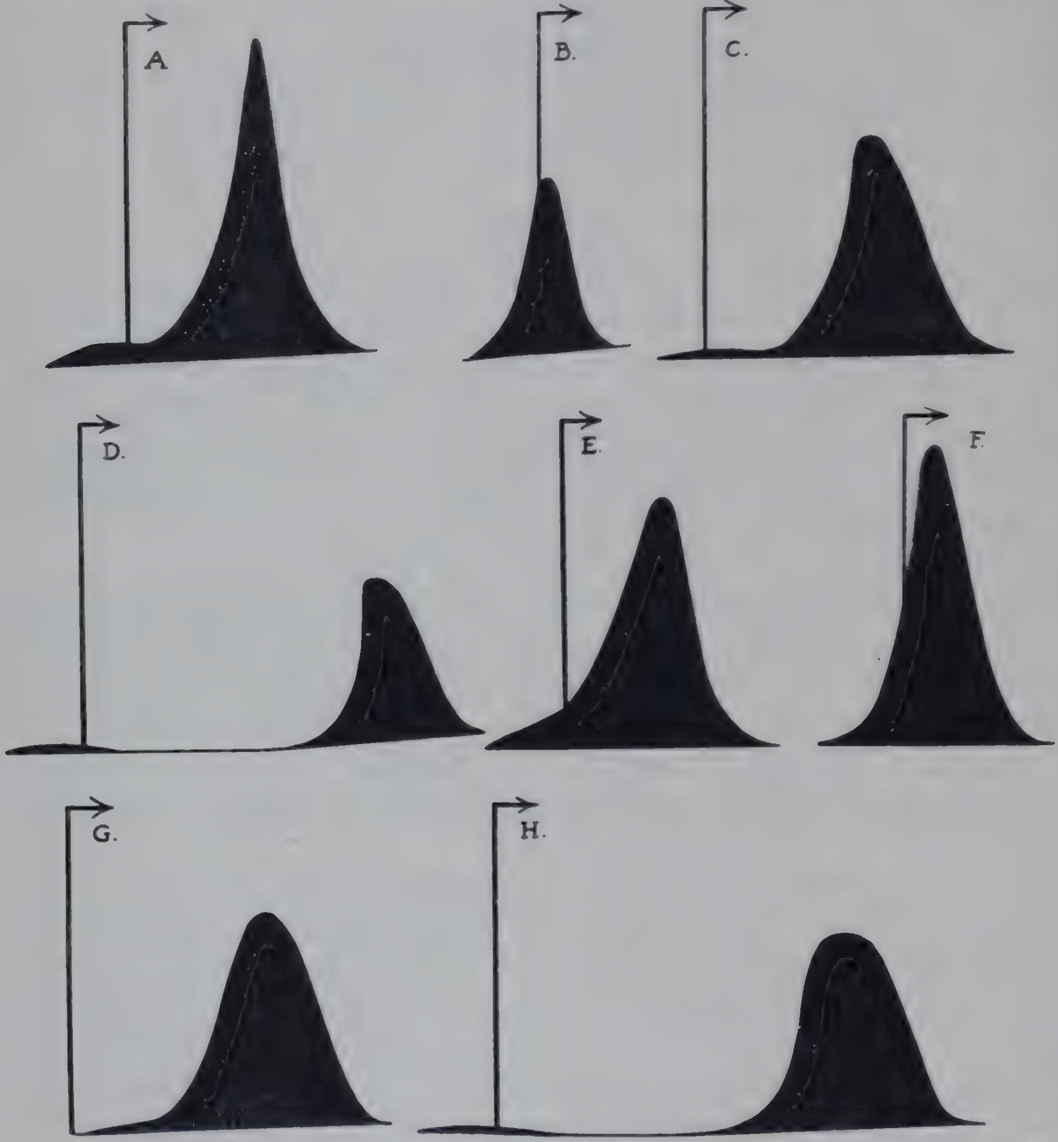


Fig. 1. Descending patterns obtained on electrophoresis of human γ-globulins. The protein concentration was about 1.0 per cent, except for *B* (0.3 per cent) and *D* (0.4 per cent). The photographs were taken at 250 minutes, with the exception of *B*, which was taken at 167 minutes. The II-3 globulin is shown in *A*, veronal buffer, pH 8.55; in *B*, cacodylate, pH 7.25; in *C*, acetate, pH 5.14; and in *D*, acetate, pH 3.43. The II-1,2 globulin is shown in *E*, veronal, pH 8.47; in *F*, cacodylate, pH 6.79; in *G*, acetate, pH 4.15; and in *H*, acetate, pH 3.44.

We were unable to detect the presence of any other serum proteins in these preparations. However, some of the patterns show greater symmetrical spreading than would be expected for electrophoretically homogeneous particles. It is also evident that the patterns obtained at acid pH values are not symmetrical, giving further indication of the inhomogeneity of these γ-globulins. The electrophoretic properties of human γ-globulins have also been recently studied by Alberty (9).

In Fig. 2 the electrophoretic mobilities are shown as a function of pH for both of the γ-globulins as obtained from descending migrations at an ionic strength of 0.1. The apparent isoelectric point of the II-1,2 fraction is at pH 7.3 and of the II-3 fraction at pH 6.85. These values are consistent with the previously recorded differences in the basic amino acid content of these proteins (8).

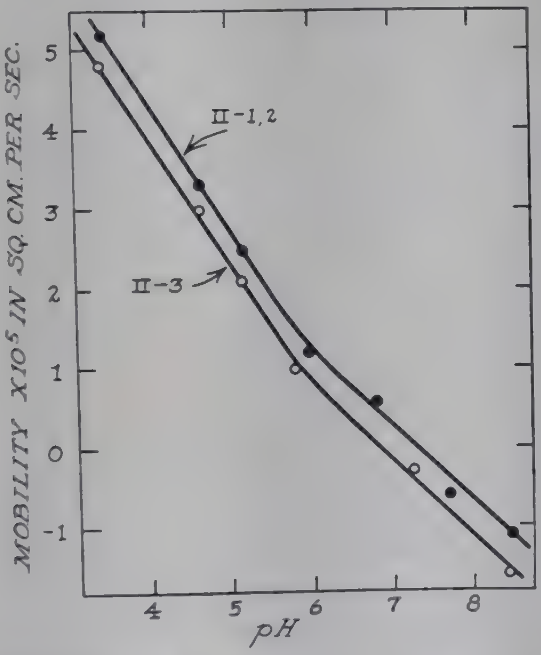


FIG. 2. Electrophoretic mobility as a function of pH for human γ-globulins II-1,2 and II-3. All of the measurements were determined from descending migrations at 1° in univalent buffers at an ionic strength of 0.1. The pH measurements were made with a glass electrode at 25°.

Human γ-globulins prepared by alcohol fractionation methods have been found to be inhomogeneous in the ultracentrifuge (10). We have made some studies with the preparations used in this investigation and have obtained results that are in agreement with previous observations. The II-1,2 and II-3 preparations contain about 75 per cent of a component with S_{20} about 6.5 to 7.0 Svedberg units, and 25 per cent of heterogeneous heavier material which sediments over a range of about 9 to 17 Svedberg units. No material lighter than the principal component was found in either preparation. Observations were made on 1 per cent protein solutions in 0.15 M NaCl at pH 7.0 or in 0.1 M veronal buffer at pH 8.5. The instrument was the electrically driven ultracentrifuge manufactured by the Specialized Instruments Corporation of Belmont, California.

Immunological Observations

Material and Methods—Female rabbits were immunized with alum-precipitated suspensions of γ-globulin fractions II-1,2 and II-3. The animals received 0.5 to 2.0 mg. of antigen intravenously four times a week for 4 to 6 weeks. For the quantitative precipitin determinations, 0.5 ml. of heat-inactivated antisera (56° for 30 minutes) was placed in a 10 ml. centrifuge tube. Antigen in 0.5 ml. of 0.15 M NaCl was then added, the fluid was mixed thoroughly, and kept at 4° for 48 hours. The precipitate was centrifuged for 30 minutes at 2000 R.P.M. with a maximum temperature of 7° in the tube. The sediment was drained and then washed with 3.0 ml. of chilled 0.15 M NaCl. This was immediately centrifuged and again drained.[1] The protein content of the precipitate was determined by the tyrosine method described by Heidelberger and MacPherson (11). The standard tyrosine curve was constructed from spectrophotometric measurements of γ-globulin II-1,2 in known quantities, as determined by a micro-Kjeldahl technique. Values for antigen are expressed as mg. of *protein* per ml. of fluid. The protein content of the precipitate, in mg., represents the quantity produced by addition of 1 ml. of antiserum to 1 ml. of antigen. For estimation of the γ-globulin content of human serum by the immunological technique, it was necessary to dilute the serum 1:30 to 1:250 before addition of the standardized antiserum. The supernatants always contained an excess of antibody. Duplicate determinations of the protein content of the precipitate were performed.

Immunological Homogeneity of γ-Globulins—In Table I the quantitative estimation of the precipitate formed by addition of varying quantities of fractions II-1,2 and II-3 to their homologous antisera is presented. The supernatant fluid was examined for presence of excess of antigen by addition of antiserum and for excess of antibody by addition of antigen. Table I also includes the calculated values for the antibody expected from the quantity of antigen added, with Equations 3 and 6 of Heidelberger and Kendall (5). Equation 3 may be expressed: $(Ab/An) = (2R - R^2/A(An))$

---

[1] We have found that a single washing of the precipitate is sufficient to remove the proteins of the supernatant fluid under the conditions specified when the volume of wash fluid is extremely high as compared to the minute quantity of precipitate. In tests where an additional washing was performed, only a negligible color reaction was given by the wash fluid.

where Ab is antibody and An is antigen in mg. A represents the total units of reactive antibody in the system and R represents the value for Ab/An at the zone of equivalence, where there is no free antigen or antibody in the system. By plotting the experimentally observed values for Ab/An against An, a straight line is obtained for pure antigens. The intercept of the line on the ordinate equals $2R$ and the slope is represented by $-R^2/A$. From these values, it is possible to calculate the amount of antibody which may be obtained by the addition of a known quantity of antigen.

TABLE I

Quantitative Precipitation Data with Homologous Antigen and Antibody of Human γ-Globulin

Antigen No.	Amount of antigen	Total ppt.	Antibody			Antibody / antigen observed	Supernatant	
			Observed	Calculated (Equation 3)	Calculated (Equation 6)		Antigen	Antibody
	mg.	*mg.*	*mg.*	*mg.*	*mg.*			
II-1,2	0.10	1.0	0.9	0.9	0.9	8.7	4+	−
	0.30	2.5	2.2	2.3	2.3	7.3	4+	−
	0.50	4.1	3.6	3.5	3.4	7.2	4+	−
	0.60	4.4	3.8	3.9	3.8	6.3	4+	−
	0.75	5.7	4.9	4.5	4.4	6.4	4+	−
	0.85	5.7	4.9		4.8	5.7	+	−
	0.95	5.9	4.9		4.9	5.2	−	−
	1.05	5.6	4.6				−	+
II-3	0.10	1.0	0.9	0.9	0.9	9.0	4+	−
	0.20	1.7	1.5	1.6	1.6	7.6	4+	−
	0.30	2.3	2.0	2.2	2.1	6.7	4+	−
	0.40	3.0	2.6	2.6	2.6	6.5	4+	−
	0.50	3.6	3.1	3.0	2.9	6.2	++	−
	0.60	3.8	3.2	3.2	3.2	5.3	+	−
	0.60*	4.0	3.4	3.2	3.2	5.7	+	−
	0.70*	4.4	3.7		3.4	5.2	−	−
	0.80*	4.8	4.0				−	+

* Serum + antigen stood 96 hours instead of 48 hours in the cold.

Equation 6 is expressed as follows: $Ab/An = 3R'' - 2[(R'')^3 An/A]^{\frac{1}{2}}$. The symbols have the same meaning as in the previous equation. Equations 3 and 6 are valid only in the region of antibody excess. It will be observed that the calculated values obtained from these equations are consistent with the observed values for both the II-1,2 and II-3 globulins with the homologous antisera (Table I). The same results were obtained with four different antisera to II-1,2 globulin and three antisera to II-3 globulin.

It should be noted that, with both antigens, the supernatant did not contain antigen and antibody simultaneously. This constitutes one criterion for immunological homogeneity (12). In Fig. 3 are presented examples of findings when "antibody/antigen" is plotted against antigen. Within the limits of the experimental error, straight lines were obtained. These results offer further evidence that the antigens behave as immunologically homogeneous proteins.

Immunological Equivalence of γ-Globulins II-1,2 and II-3—When antiserum to fraction II-1,2 was treated with an excess of fraction II-3, it failed to react with II-1,2, the homologous antigen. The same finding was obtained with antiserum to fraction II-3 when it was absorbed with II-1,2 antigen. In Table II quantitative comparative studies of the antigens are given. The amounts of precipitate recovered by addition of equal quantities of II-1,2 and II-3 antigens to II-1,2 antiserum are presented. Within the limits of experimental error, the precipitates in the region of antibody excess were equivalent. The addition of both antigens to II-3 antiserum gave similar results.

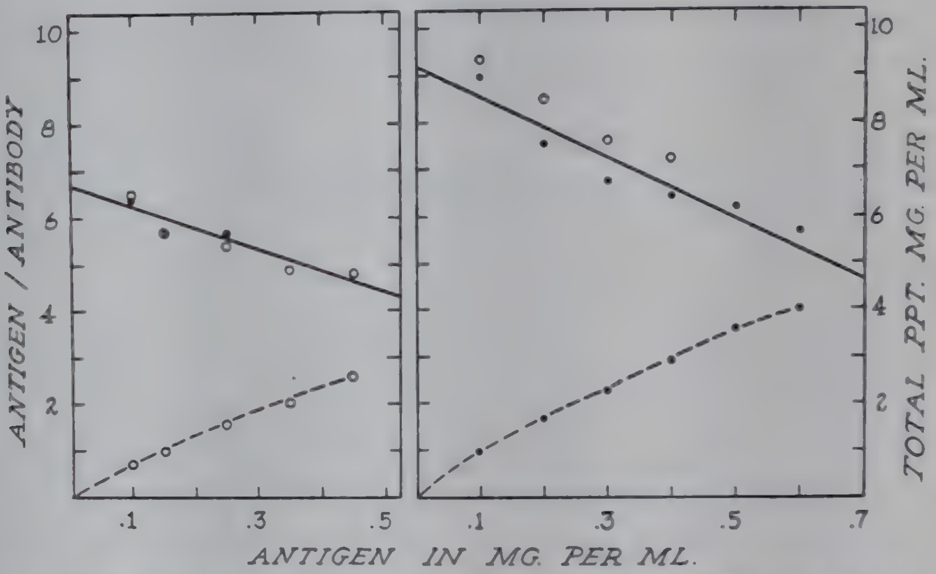


FIG. 3. The ratio of antibody to antigen as a function of antigen added. These data are presented with solid lines expected from theory for homogeneous proteins. The values on the left are for an antiserum to the II-1,2 globulin, and those on the right for an antiserum to the II-3 globulin, ○, II-1,2 antigen, ●, II-3 antigen, for both experiments; the solid lines are drawn to fit the values for the homologous antigen; the dash lines give the total precipitates obtained with different amounts of antigen; the ordinate values are given on the extreme right.

In Table III are presented the quantities of antigen in several human sera which precipitated with various rabbit antisera to fractions II-1,2 and II-3. A curve was constructed for each antiserum by adding known quantities of homologous antigen and determining the total precipitate formed. Greatly diluted human sera were added to the standardized antisera. The amount of reactive antigen in the human sera, within the zone of antibody excess, was then calculated from the curves. Within the limits of error which were necessarily great because of the enormous dilutions of the human serum antigens, the values with various II-1,2 and II-3 antisera appear comparable.

TABLE II

Quantity of Precipitate Obtained with II-1,2 and II-3 Antigens Added to Homologous and Heterologous Antisera

Antiserum No.	Animal No.	Antigen added	Quantity of ppt.	
			Antigen II-1,2	Antigen II-3
		mg. per ml.	*mg. per ml.*	*mg. per ml.*
II-1,2	W-4	0.10	0.8	0.7
II-1,2	W-4	0.15	1.0	1.0
II-1,2	W-4	0.25	1.6	1.7
II-3	W-7-I	0.10	1.0	0.8
II-3	W-7-I	0.15	1.5	1.5
II-3	W-7-I	0.25	2.0	1.7
II-3	W-7-II	0.10	1.0	1.0
II-3	W-7-II	0.20	1.9	1.7
II-3	W-7-II	0.30	2.6	2.3
II-3	W-7-II	0.40	3.3	3.0

TABLE III

Estimation of Immunological γ-Globulin by Using Various Antisera with Human Serum As Antigen

The amount of precipitate is given as gm. of protein per 100 ml. of human serum. The various antisera are differentiated by the rabbit identification numbers and the antigen in parentheses used for immunization.

Human serum No.	γ-Globulin calculated in ppt.				
	Rabbit W-16-I (II-1,2)	Rabbit W-16-II (II-1,2)	Rabbit W-4 (II-1,2)	Rabbit W-7-I (II-3)	Rabbit W-7-II (II-3)
1	2.4	2.7	2.4	2.8	2.5
2	2.3	2.5		2.2	2.1
3		1.0	1.0	1.1	
4	0.7	0.8		1.1	
5		2.1	2.2	2.0	2.0

Immunological Estimation of γ-Globulin in Human Sera—By using the technique just described above, the total quantity of reactive antigen to

γ-globulin antiserum was estimated for fifteen normal human sera. The values ranged from 1.02 to 2.29 gm. per 100 ml. of sera (13 to 32 per cent of the total serum protein). The mean value was 1.74 gm. or 24 per cent of the total serum protein. The corresponding electrophoretic values for the total γ-globulin in the fifteen sera ranged from 0.30 to 1.25 gm. per 100 ml. of serum (4 to 17 per cent of the total serum protein). The mean value was 13 per cent or 0.90 gm. per 100 ml. In Fig. 4, comparative immunological and electrophoretic estimations for γ-globulin, expressed as per cent of total serum protein, are given for fifteen normal sera and for nine abnormal sera. It will be noted that the results for γ-globulin obtained immunologically are considerably higher than the electrophoretically determined values for γ-globulin. In fact, there appears to be

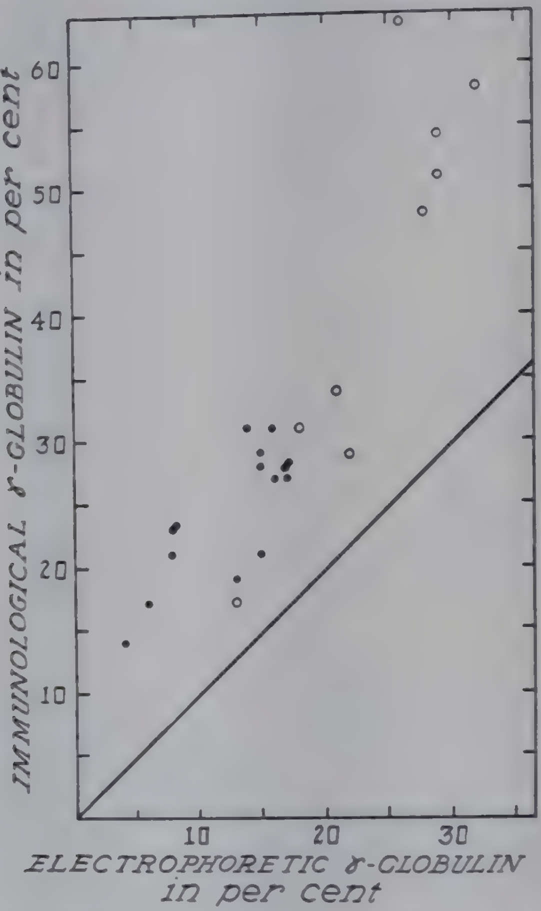


FIG. 4. Electrophoretic estimation of serum γ-globulin as compared with the immunological values. The results are expressed as per cent of the total serum protein; ○, abnormal sera, ●, normal sera. The line indicates the theoretical expectation if the two methods give concordant results. The electrophoretic estimations were made at 1.5 per cent protein concentration from descending migrations in veronal buffer at pH 8.4 to 8.6 for 250 minutes. The values are the sum of both γ_1 and γ_2 components.

roughly 10 per cent additional serum protein which reacts immunologically as γ-globulin.

DISCUSSION

The immunological homogeneity of the two γ-globulin fractions II-1,2 and II-3 is somewhat surprising, since both preparations are inhomogeneous with respect to electrical charge and size, and since they are known to contain a mixture of antibodies (13). However, the globulin fraction isolated by Kendall (3) was also found to be homogeneous immunologically. The immunological homogeneity of the two preparations provides further evidence that the particles of different size observed in the ultracentrifuge truly represent γ-globulins and not unrelated extraneous proteins (10). Somewhat more unexpected is the apparent immunological equivalence of the two preparations in spite of their differences in solubility, electrophoretic mobility, and chemical composition.

It should be emphasized that, in our study, the amounts of antigen employed for production of antisera were small and the antibody titers were low. With larger quantities of antigen for immunization, less specific antibodies may be formed. Also, slight impurities present in the immunizing antigen may give rise to other antibodies. For example, Deutsch *et al.* (14) found that rabbit antiserum prepared by three intraperitoneal injections of 100 mg. of γ-globulin also reacts with human albumin. No such cross-reaction occurred with our antisera. Kabat and Heidelberger (15) found that antiserum to crystalline horse serum albumin reacts with homologous antibody as a pure antigen only when the rabbit antiserum is prepared with small quantities of immunizing antigen.

Our finding of 1.02 to 2.29 gm. of γ-globulin per 100 ml. of human serum as estimated immunologically is in excellent agreement with Kendall's values (3) of 1.1 to 2.1 gm. by using antisera to his globulin. This agreement is particularly noteworthy since Kendall's globulin was prepared by salt fractionation, whereas the proteins used in our study were isolated by an alcohol precipitation method (7). The immunological values are considerably in excess of the electrophoretic estimations (Fig. 4), although we have included in our electrophoretic γ-globulin values the sum of the γ_1 and γ_2 components identified in normal human serum by Deutsch, Alberty, and Gosting (16). The γ_1-globulin is generally obscured by the fibrinogen peak in plasma.

It is evident that the antibody to γ-globulin reacts not only with the γ-globulin which is estimated electrophoretically, but with an additional serum constituent. Obviously, immunological evaluation of γ-globulin will give higher results than the electrophoretic estimations. Nevertheless, the immunological determinations may be useful in selected instances for the study of abnormal sera and other body fluids (6).

Although the material which cross-reacts immunologically with γ-globulin is still unknown, certain of its characteristics can be described. It is not albumin since our antisera did not react with human albumin. Therefore, the substance must be present in one of the globulin fractions. In some of the sera, it is present in larger amounts than the electrophoretically estimated γ-globulin, and must constitute a large part of the α- or β-globulins.

The apparent cross-reaction between γ-globulin antisera and some other component of human serum is not the first instance in which this has been found. Treffers, Moore, and Heidelberger (17) demonstrated that horse β-globulin can react with antisera to horse γ-globulin. Although the human γ-globulins apparently contain most of the known antibodies to disease (13), there is evidence that the T- and γ-globulins of the horse (18) and the cow[2] do not possess all of the antibody activities found in the sera of hyperimmunized animals of these species. Our finding that protein immunologically related to γ-globulin occurs in the α- or β-globulins suggests that this related protein also may be capable of possessing antibody activity in the human.

SUMMARY

1. The human γ-globulin fractions II-1,2 and II-3 possess isoelectric points at pH 7.3 and 6.85, respectively. Although no other serum constituents could be detected electrophoretically in these fractions, both show some evidence of electrical inhomogeneity. Likewise, neither fraction is homogeneous in the ultracentrifuge.

2. Both γ-globulins (II-1,2 and II-3) behave as homogeneous antigens towards rabbit antisera, thus providing additional evidence that the particles of different electrophoretic mobility and size are truly γ-globulins. The two fractions appear to be immunologically equivalent in spite of their known differences in other properties.

3. Antisera to human γ-globulin react with some additional, and as yet unidentified, globulin component of human serum. This yields abnormally high values for immunologically determined γ-globulin when compared with the electrophoretic estimations.

BIBLIOGRAPHY

1. Dale, H., and Hartley, P., *Biochem. J.*, **10**, 408 (1916).
2. Harris, T., and Eagle, H., *J. Gen. Physiol.*, **19**, 383 (1935–36).
3. Kendall, F. E., *J. Clin. Invest.*, **16**, 921 (1937).
4. Cohn, E. J., McMeekin, T. L., Oncley, J. L., Newell, J. M., and Hughes, W. L., *J. Am. Chem. Soc.*, **62**, 3386 (1940).

---

[2] Unpublished observations by E. L. S.

5. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, **62**, 697 (1935).
6. Kabat, E. A., Glusman, M., and Knaub, V., *Federation Proc.*, **7**, 306 (1948); *Am. J. Med.*, **4**, 653 (1948).
7. Oncley, J. L., Melin, M., Richert, D. A., Cameron, J. W., and Gross, P. M., *J. Am. Chem. Soc.*, in press.
8. Smith, E. L., Greene, R. D., and Bartner, E., *J. Biol. Chem.*, **164**, 359 (1946). Smith, E. L., and Greene, R. D., *J. Biol. Chem.*, **171**, 355 (1947).
9. Alberty, R. A., *J. Am. Chem. Soc.*, **70**, 1675 (1948).
10. Williams, J. W., Petermann, M. L., Colovos, G. C., Goodloe, M. B., Oncley, J. L., and Armstrong, S. H., *J. Clin. Invest.*, **23**, 433 (1944). Oncley, J. L., Scatchard, G., and Brown, A., *J. Phys. and Colloid Chem.*, **51**, 184 (1947).
11. Heidelberger, M., and MacPherson, C. F. C., *Science*, **97**, 405 (1943); **98**, 63 (1943).
12. Kabat, E. A., *J. Immunol.*, **47**, 513 (1943).
13. Enders, J. F., *J. Clin. Invest.*, **23**, 510 (1944).
14. Deutsch, H. F., Alberty, R. A., Gosting, L. J., and Williams, J. W., *J. Immunol.*, **56**, 183 (1947).
15. Kabat, E. A., and Heidelberger, M., *J. Exp. Med.*, **66**, 229 (1937).
16. Deutsch, H. F., Alberty, R. A., and Gosting, L. J., *J. Biol. Chem.*, **165**, 21 (1946).
17. Treffers, H. P., Moore, D. H., and Heidelberger, M., *J. Exp. Med.*, **75**, 135 (1942).
18. Smith, E. L., and Gerlough, T. D., *J. Biol. Chem.*, **167**, 679 (1947).

THE BIOCHEMISTRY OF THE METABOLIC FECAL PROTEIN NITROGEN

By ANTHONY A. ALBANESE, VIRGINIA I. DAVIS, MARILYN LEIN, AND EMILIE M. SMETAK

(From the Department of Pediatrics, New York University College of Medicine, and the Children's Medical Service, Bellevue Hospital, New York)

(Received for publication, June 9, 1948)

In determining the nutritional value of various protein preparations in the infant in terms of nitrogen balance and growth response, it occurred to us that these results would gain in significance if the nature of the fecal proteins excreted under the various regimens were known. Although it is clear from the literature that the amount and composition of the fecal fat are to a large extent independent of the dietary fat (1), we were unable to find any clear evidence on the effect of dietary proteins on the composition of the fecal proteins (2). The attempt to assess the nature of this relationship raised the problem (*a*) of finding a suitable method for the quantitative determination of fecal proteins and (*b*) of isolating suitable quantities of these proteins for amino acid analysis. Heretofore only qualitative or indirect quantitative (3) procedures have been employed for the estimation of fecal proteins.

After a number of preliminary experiments we found that the fecal protein could be determined quantitatively by a micro-Kjeldahl analysis of the proteins precipitated isoelectrically at pH 6.0 from an alkaline ethanol extract of an aqueous homogenate of the feces. The analytical adequacy of this technique was further demonstrated by recovery tests and by its failure to measure proteins of the intestinal flora. Application of the method to the stools of ten infants on various diets for periods ranging from 1 to 16 weeks revealed that an average of 22.4 per cent of the fecal N arises from the protein moiety and that fluctuations from this mean value are spontaneous and not related to the diet.

Analyses of fecal proteins isolated by a modification of the an alytical procedure from the stools of these infants disclosed that the amino acid composition of these products was unaffected by the use of whole milk proteins, casein-, tryptophan-, isoleucine-, or lysine-deficient preparations as the nitrogen component of the diet. This observation, the absence of bacterial bodies from the protein fraction, and the uniformity of amino acid pattern of the different fecal protein specimens suggest that this stool fraction is not derived from residues of the dietary N or from the bodies of the intestinal bacteria, but from definite intestinal secretions.

EXPERIMENTAL

Exploratory tests disclosed that the fecal protein could be isoelectrically precipitated from the alkaline alcoholic extracts by adjustment of the pH to 6.0 ± 0.1 with HCl, H_2SO_4, or trichloroacetic acid. The isoelectric point of this protein is to be compared with that of casein, 4.6, and lactalbumin, 4.5 to 5.5 (4). The protein can also be precipitated by half saturation of the extract at pH 7.0 with ammonium sulfate. The negative Exton, biuret, and trichloroacetic acid tests, given by filtrates from the isoelectric or salt precipitation of the protein, indicated that either of these two procedures could be employed in the quantitative measurement of the fecal protein N.

Determination of Fecal Protein N—24 hour stools were collected in wide mouth preserving jars containing 40 cc. of 70 per cent ethyl alcohol. Prior to removing samples for analysis the volume of the mixture was adjusted to 200 cc. with water and homogenized with a mechanical stirrer. (5 day pools were collected in 200 cc. of 70 per cent ethanol and made to 1 liter with water before analysis.) To duplicate 10 cc. aliquots of the homogenate, which were transferred into 40 cc. graduated centrifuge tubes by means of a wide tipped Mohr pipette, were added 1 cc. of 10 per cent NaOH and 95 per cent ethanol to the 20 cc. mark. These specimens were then stirred mechanically for 5 minutes with an air-driven stirrer and centrifuged for 10 minutes at 3000 R.P.M. A clear supernatant fluid was usually obtained; if it remained cloudy it was clarified by filtering through fluted Whatman paper No. 12. To 10 cc. aliquots of the clear fluid in 15 cc. centrifuge tubes were added 5 drops of brom-cresol purple indicator (0.02 per cent alcoholic solution) and the pH adjusted to 6.0 with 40 per cent trichloroacetic acid (about 0.7 cc. is required). The reaction mixture was stored in the refrigerator for 30 minutes and then centrifuged. The supernatant fluid was discarded and the precipitated protein was washed by resuspension in 5 cc. of cold 5 per cent trichloroacetic acid, centrifugation, and decantation of the supernatant fluid. The washed protein precipitates were then transferred quantitatively to 100 cc. Kjeldahl flasks with the aid of a minimum amount of water and the nitrogen content was determined by the micro-Kjeldahl procedure of Meeker and Wagner (5). The analytical adequacy of the single extraction was demonstrated by the absence of protein in a second alkaline alcohol extract of the first extraction residues. The total N of the stool suspension was determined by direct micro-Kjeldahl analysis of 10 cc. aliquots of the homogenate.

Isolation and Analysis of Fecal Protein—In order to avoid contamination of the fecal protein by residues of a previous dietary regimen, the various diets were fed for 2 or more successive weeks and the fecal proteins isolated from the feces collected during the last week of the diet period.

Aliquots of the 5 day pools of two or three infants, sufficient to make a total volume of 2 liters, were employed for each preparation. These samples were combined in a 5 liter round bottom flask and treated as described above with a proportionate increase of all the reagents. The final reaction mixture was stored overnight in a refrigerator at 4°. The precipitated protein was collected by decantation of the supernatant liquid, purified by electrodialysis, collected by centrifugation, and granulated by successive suspension in 50, 75, and 95 per cent acetone. This product was dried in a stream of compressed air and then in a 37° oven for 24 hours. The total N, moisture, and ash contents of each sample were determined.

Subsequently acid and alkaline hydrolysates of these proteins were prepared. The alkaline hydrolysate was employed for the determination of tryptophan by the procedure described by Albanese and Frankston (6). The other amino acids were determined in hydrolysates prepared by refluxing 1.5 gm. of the proteins with 10 cc. of 6 N HCl for 24 hours. The total N content of the hydrolysate was determined directly by micro-Kjeldahl analysis; then the excess of acid was removed by concentration *in vacuo* and the humin was separated by filtration. The volume of this filtrate was adjusted to 50 cc. and appropriate samples were removed for the various amino acid analyses.

Metabolism Experiments—The fecal specimens employed in this study were collected from normal healthy male infants who were given the various diets in five feedings daily at the rate of approximately 100 calories per kilo of body weight and 500 mg. of ascorbic acid together with 15 drops of oleum percomorphum daily. The diet periods were of 7 days duration and consecutive, but excreta collections were omitted on week-ends to avoid complications which might arise from the continued use of restraints. The subjects were immobilized by abdominal restraints which were also designed to hold the urinary adapters in place. 24 hour urine specimens were collected in bottles containing 10 cc. of 15 per cent (by volume) HCl and 1 cc. of 10 per cent alcoholic thymol.

The feces were collected in 19 cm. porcelain evaporating dishes which were held in place by a properly shaped excavation in the mattress and the daily stools were accumulated under refrigeration for each period in jars containing 200 cc. of 70 per cent ethanol. The subjects were weighed daily during the course of the investigation.

The synthetic diets fed in this experiment contained approximately 100 calories per 100 gm., the percentage caloric distribution in all instances being as follows: protein 14, fat 36, carbohydrate 50. The composition of the synthetic diets is shown in Table I. The evaporated milk formula employed in the control experiments (Table IV) had the following composition: evaporated milk 40 cc. (55.3 calories), corn syrup 6 cc. (17.7 calories), water 54 cc. This supplied approximately 73 calories per 100 cc. and the nitrogen content of each batch was controlled by micro-Kjeldahl analysis.

The data on nitrogen retention were calculated from the results of nitrogen determinations of the 24 hour urine collections, analysis of the pooled feces for each period, and from computation of the daily nitrogen intake based on food consumption records and the known nitrogen content of the diets.

TABLE I

Composition of Diets

All diets were fed at the rate of 100 calories and 3.5 gm. of protein (N × 6.25) per kilo of body weight.

	Diets				
	Casein	Gluten	TH	CTH	BHb
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
Casein (Sheffield)	3.5	0	0	0	0
Gluten (Interchemical)	0	3.5	0	0	0
Acid-hydrolyzed casein*	0	0	3.5	3.4	0
" beef hemoglobin	0	0	0	0	3.4
L-Tryptophan	0	0	0	0.06	0.06
L-Cystine	0	0	0	0.04	0.04
Brewers' yeast	1.0	1.0	1.0	1.0	1.0
Olive oil	4.0	4.0	4.0	4.0	4.0
Dextri-Maltose No. 2†	9.6	9.6	9.6	9.6	9.6
Arrowroot starch	2.3	2.3	2.3	2.3	2.3
Salt mixture‡	1.6	1.6	1.6	1.6	1.6
Water	78.0	78.0	78.0	78.0	78.0
Total	100.0	100.0	100.0	100.0	100.0
		mg.	*mg.*	*mg.*	*mg.*
Estimated content of deficient amino acid		52	6	59	36

* N × 6.25 = gm. of protein.

† Kindly supplied by Mead Johnson and Company.

‡ The salt mixture employed had the following composition (measured in gm.): $FeSO_4$ 0.9, NaCl 6, calcium gluconate 48, $Ca(OH)_2$ 12, KH_2PO_4 7, KCl 6, MgO 0.1.

Results

The efficacy of the analytical procedure described previously was evaluated by recovery tests in which various biological products were added to the fecal homogenates. It is clear from these experiments (Table II) that the quantitative isoelectric precipitation of fecal protein is not influenced by the presence of the proteins of cow's milk or human plasma, non-protein nitrogenous substances, or the nitrogen of bacterial bodies.

The cultures of *Escherichia coli* employed in these tests were grown in the medium defined by Sayhun (7), dialyzed free of all unorganized N, and concentrated 5-fold by centrifugation. Quite apart from the present purpose, it is of interest to note that micro-Kjeldahl analysis of the three fractions derived by the application of our procedure to fecal homogenates gave the following percentage nitrogen distribution: protein 27.5, protein-free extract 31.3, and residue (consisting in part of bacterial bodies) 41.2.

Effect of Diet on Fecal Protein Output—The data obtained by the application of the method to the stools of infants fed a Dextri-Maltose-modified evaporated milk formula are shown in Table III. Examination of these data reveals that the amount of protein occurring in the feces is subject to considerable individual variation. By the use of the protein factor (N × 6.25) it appears that 0.4 to 1.2 gm. of fecal protein are excreted daily and that the amount excreted is unrelated to the N retention or body weight of the infant.

TABLE II

Recovery of Fecal Proteins from Infant Stools in Presence of Added Biological Products

Fecal sample and protein N content	Source of added N	Nitrogen added	Total protein N found	Recovery of added N
		mg.	*mg.*	*per cent*
10 cc. Feces A, 1.25 mg.	Cow's milk	1.16*	1.25	0.0
10 " " B, 0.37 "	Human plasma	2.18*	0.37	0.0
10 " " C, 3.20 "	Urine	1.78	3.20	0.0
10 " " C, 3.20 "	Amigen	2.09	3.20	0.0
5 " " D, 2.55 "	*E. coli* culture	0.62	2.55	0.0

* These values were obtained by micro-Kjeldahl analysis of the protein precipitated by trichloroacetic acid treatment of the biological fluids tested.

The results of determinations of the fecal protein output of infants fed various synthetic diets are given in Table IV. These data reveal that the amount of fecal protein is not greatly influenced by the dietary N. Indeed the formation and excretion of the protein in normal amounts on diets lacking in tryptophan or isoleucine lead one to the conclusion that the fecal protein of the infant is an endogenous metabolite and not a dietary residue.

Effect of Diet on Composition of Fecal Protein—In order to inquire further into the nature of the fecal protein it was isolated from the stools collected during the various diet periods as described above. The yield of purified protein varied from 1.05 to 3.46 gm. per liter of homogenate. All of the protein samples analyzed were obtained by isoelectric precipitation at pH 6.0 ± 0.1. These were uniformly insoluble in water, but readily soluble in 10 per cent NaOH, 95 per cent formic acid, and 40 per cent urea solution, and contained no carbohydrate component. Moreover, all purified specimens of the isolated protein showed no proteolytic activity towards casein (8) and no arginase activity towards D-arginine (9). The results of amino acid analysis of the various specimens of the protein are collected in Table V. On the basis of the agreement of the values for ten of the amino

Table III

Fecal Protein Nitrogen Output of Male Infants Fed Evaporated Milk

All the results are expressed as daily averages.

Subject	Age	Body weight	Period I					Period II				
			Nitrogen retained	Total fecal N	Fecal protein N	Fecal protein N of total N	Fecal protein N of retained N	Nitrogen retained	Total fecal N	Fecal protein N	Fecal protein N of total N	Fecal protein N of retained N
	mos.	*kg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*
De.	3	4.6	790	400	72	18.0	9.1	870	350	61	17.5	7.0
Ja.	4	4.0	520	400	69	17.3	13.3	690	470	98	21.1	14.2
Ma.	8	8.5	1000	560	180	32.4	18.0	840	640	208	32.2	24.8
Sh.	2	3.3	1050	450	111	24.8	10.6	840	400	87	21.9	10.4
Od.	9	8.5	1030	440	70	15.7	6.8	1380	280	77	21.6	5.6
Sm.	4	3.6	860	320	75	23.2	8.7	950	300	61	20.1	6.9

Table IV

Effect of Various Diets on Fecal Protein Nitrogen Output of Male Infant

All the results are expressed as daily averages.

Subject	Age	Body weight	Diet	Nitrogen retained	Total fecal N	Fecal protein N of total fecal N	Fecal protein N of retained N
	mos.	*kg.*		*mg.*	*mg.*	*per cent*	*per cent*
Sa.	7.5	6.3	Casein	1090	143	23.5	3.1
Go.	14.0	6.8	"	530	151	44.5	12.6
Ge.	25.7	9.6	"	2360	204	28.8	2.5
Sa.	9.5	7.1	CTH	1890	160	19.7	1.6
Go.	16.0	7.3	"	470	230	17.1	8.3
Ge.	27.7	9.9	"	1150	304	36.7	9.6
Go.	16.5	7.4	TH	260	412	15.8	25.0
Ge.	28.2	10.0	"	320	304	12.7	11.9
Od.	11.0	8.2	BHb	550	314	20.7	11.8
Ja.	7.2	6.0	"	420	416	23.6	23.0
Ca.	3.0	6.5	"	320	292	23.3	21.0
De.	7.0	5.6	Gluten	560	340	38.8	23.2
Ja.	4.2	4.8	"	540	418	35.3	27.4

acids and previously mentioned characteristics, it would seem reasonable to assume that the different samples represent a single protein. The possibility of a dissimilarity of pattern for the remaining amino acids, of course, exists, but this possibility is contraindicated by the uniformity of the organic acid: amino N ratios found for the different samples. The lysine content of the protein, 12.2 per cent, is uncommonly high (21). Finally, the presence of tryptophan, isoleucine, and lysine in the fecal protein isolated from the stools of infants fed hydrolysates deficient in these amino acids should dispel any further doubts regarding the dietary origin of the fecal protein.

TABLE V

Analysis of Fecal Protein Isolated from Feces of Infants Fed Various Diets

The amino acid values are given as per cent amino acid N of total N.

	Diet					
	Evaporated milk	Casein	CTH	TH	BHb	Gluten
Moisture content, %	7.63	7.27	7.55	8.88	7.27	3.85
Ash content, %	0.15	0.14	0.19	0.17	0.17	0.11
Corrected N content, %	14.65	14.72	14.52	14.43	14.59	14.67
Ratio, organic acid (10)* to amino N†	0.65	0.67	0.69	0.69	0.69	0.67
Amino N (11)	80.6	81.90	82.2	81.3	82.5	81.6
Threonine (12)	6.0	6.1	5.8	5.9	5.8	5.7
Arginine (13)	12.7	12.9	12.7	12.7	12.6	12.8
Histidine (14)	5.4	5.6	5.2	5.2	5.7	5.7
Lysine (15)	16.4	16.0	16.3	16.6	16.2	16.1
Cystine (16)	1.3	1.2	1.3	1.2	1.4	1.3
Methionine (17)	1.9	2.0	1.8	1.9	1.9	1.8
Isoleucine (18)	4.6	4.7	4.6	4.4	4.3	4.2
Phenylalanine (19)	2.9	3.0	3.1	3.3	3.1	2.8
Tyrosine (20)	2.1	2.3	2.0	2.3	2.2	2.0
Tryptophan (6)	3.1	3.2	3.3	3.4	3.3	3.4

* The figures in parentheses represent bibliographic references.

† This ratio was obtained by dividing the acid titer (expressed as cc. of 0.1 N HCl) by mg. of amino N of the sample.

Comments

In 1892 Voit (21) found that the excretion of substances from an isolated loop of the intestine of dogs produced a mass similar in constitution and nitrogen content to that produced in the normal intestine of the same animal through which meat and fat were passing. He therefore concluded that the feces are derived principally from the substances excreted through the wall of the intestine and that the nitrogen so excreted is as much to be considered a product of protein metabolism as is the nitrogen of urea. He expressed regret at that time that very little was known regarding the chemistry of these nitrogenous compounds excreted into the intestine.

Subsequently Schneider (22) was able to show clearly that two distinct fractions of metabolic nitrogen exist in the feces. One fraction is constant for each animal but varies among different animals roughly in proportion to size. This is the truly excretory component and would be represented by the fecal material produced during fast. The other fraction varies in proportion to the intake of dry matter. This component is a true digestive waste. In Mitchell's opinion (23) the excretory fraction of the metabolic nitrogen is so small that on amounts of food permitting maintenance of weight or growth its effect on the ratio of total metabolic nitrogen to dry matter consumed is negligible. In a later publication Schneider (24) reported that "contrary to results on rats and pigs, the results on human subjects indicate that all of the metabolic nitrogen of human feces is proportional to food intake." This viewpoint is in contradiction to the conclusions of Thomas (25) and Martin and Robison (26), who considered all of the metabolic nitrogen of human feces to be constant.

Obviously, the existence and nature of the metabolic fecal nitrogen are a matter of considerable significance in the determination of the biological value of dietary proteins. It would appear from the observation reported here that in the infant one of the components of the metabolic fecal nitrogen is a protein. The evidence presented indicates that the amount and amino acid composition of this excreted protein do not seem to be influenced by the quantity or quality of the diet.

These findings strongly suggest that the protein which we have described is an endogenous excretory product which might be appropriately named *fecanin*. Attention is called to the fact, however, that the occurrence of proteins in the stools of infants and adults has been previously reported by other investigators who generally attributed the origin of these proteins to exogenous sources, *e.g.* food residues, end-products of internal hemorrhage, pathological exudates, and bacterial bodies (3, 27). However, since no attempt was made to isolate or characterize the fecal proteins in these previous studies, the erroneous conclusions drawn regarding the origin of the proteins are easily understandable. Moreover, the presence of dietary protein residues in the isolated protein fraction could not be excluded beyond a doubt in the present study except by the substitution of protein hydrolysates for the protein moiety of the diet. It is obvious that when poorly assimilated proteins are present in the diet or conditions of poor assimilation prevail in the organism, the differentiation of the metabolic and waste proteins in the feces may prove a difficult, if not impossible task.

The isolation and proof of identity of the metabolic fecal protein in the adult could probably be achieved under conditions of starvation or artificial alimentation. Apparently under conditions of prolonged fasting such as reported by Benedict (28) and Paton and Stockman (29) this excretion did not occur, or was so reduced in quantity as to escape measurement.

It is clear from the foregoing that the nitrogen lost in the feces in the form of fecal protein constitutes a loss of anabolic N. Although this loss constitutes an average of only 11.9 per cent of the retained N, the high content of essential amino acids of this fraction greatly augments its significance. This is particularly true when poor quality or deficient diets are fed. Furthermore, since the formation of the proteins involves a considerable biosynthetic effort on the part of the organism, the loss must affect the nitrogen economy of the organism far more than the N content of the protein would indicate. These considerations would seem to make a study of the factors concerned in the formation of the fecal protein and of its physiological functions worth while.

SUMMARY

In infants fed experimental diets it has been found that an average of 22.4 per cent of the fecal N arises from the presence of a protein which failed to reflect in its amino acid composition and other properties the dietary changes imposed on the organism from which it was derived. It is believed that this protein constitutes a moiety of the excretory or metabolic fecal N and its implications on the calculations of the biological value of dietary proteins are discussed.

We are gratefully indebted to Dr. Selma E. Snyderman for the medical care of the subjects.

BIBLIOGRAPHY

1. Lusk, G., The elements of the science of nutrition, Philadelphia and London, 4th edition, 48 (1928).
2. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Interpretations, Baltimore, **1**, 641 (1946).
3. Coope, R., *Lancet*, **1**, 1311 (1923). Becker, M., and Woldan, E., *J. Landw.*, **87**, 260 (1940).
4. Schmidt, C. L. A., The chemistry of the amino acids and proteins, Springfield, 1st edition, 618 (1938).
5. Meeker, E. W., and Wagner, E. C., *Ind. and Eng. Chem., Anal. Ed.*, **5**, 396 (1933).
6. Albanese, A. A., and Frankston, J. E., *J. Biol. Chem.*, **157**, 59 (1945).
7. Sayhun, M., Beard, P., Schultz, E. W., Snow, J., and Cross, E., *J. Infect. Dis.*, **58**, 28 (1936).
8. Frankel, E. M., *J. Biol. Chem.*, **26**, 31 (1916).
9. Albanese, A. A., Irby, V., and Frankston, J. E., *J. Biol. Chem.*, **160**, 25 (1945).

10. Van Slyke, D. D., and Palmer, W. W., *J. Biol. Chem.*, **41**, 567 (1920).
11. Albanese, A. A., and Irby, V., *J. Biol. Chem.*, **153**, 583 (1944).
12. Winnick, T., *J. Biol. Chem.*, **142**, 461 (1942).
13. Albanese, A. A., and Frankston, J. E., *J. Biol. Chem.*, **159**, 185 (1945).
14. Albanese, A. A., Frankston, J. E., and Irby, V., *J. Biol. Chem.*, **160**, 441 (1945).
15. Cavett, J. W., *J. Biol. Chem.*, **95**, 335 (1932).
16. Sullivan, M. X., and Hess, W. C., *J. Biol. Chem.*, **116**, 221 (1936).
17. Albanese, A. A., Frankston, J. E., and Irby, V., *J. Biol. Chem.*, **156**, 293 (1944).
18. Albanese, A. A., and Irby, V., *Arch. Biochem.*, **17**, 21 (1948).
19. Albanese, A. A., *J. Biol. Chem.*, **155**, 291 (1944).
20. Albanese, A. A., Irby, V., and Lein, M., *J. Biol. Chem.*, **166**, 513 (1946).
21. Voit, F., *Z. Biol.*, **29**, 325 (1892).
22. Schneider, B. H., *Biochem. J.*, **28**, 360 (1934).
23. Mitchell, H. H., *J. Biol. Chem.*, **105**, 537 (1934).
24. Schneider, B. H., *J. Biol. Chem.*, **109**, 249 (1935).
25. Thomas, K., *Arch. Anat. u. Physiol., Physiol. Abt.*, 219 (1909).
26. Martin, C. J., and Robison, R., *Biochem. J.*, **16**, 407 (1922).
27. Tsuchiga, I., *Z. exp. Path. u. Therap.*, **5**, 455 (1909). Bosworth, A. N., *Am. J. Dis. Child.*, **22**, 613 (1921). van Meeteren, A., *Nederl. Tijdschr. Geneeskunde*, **81**, 5922 (1937).
28. Benedict, F. B., *Carnegie Inst. Washington, Pub. No. 203* (1915).
29. Paton, D. N., and Stockman, R., *Proc. Roy. Soc. Edinburgh*, **16**, 121 (1888–89).

THE AMMONIA AND GLUTAMINE CONTENT OF THE BRAIN

By DEREK RICHTER AND REX M. C. DAWSON

(*From the Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff, England*)

(Received for publication, July 29, 1948)

The values given in the literature for the ammonia content of the brain show wide variation (1–3). Most of these values are of doubtful reliability, since the methods of estimation have not generally taken into account the presence of labile acid amides such as glutamine, which are now known to occur in the brain and which easily liberate ammonia on treatment with alkalies under the conditions commonly used for the estimation of ammonia (4, 5). Interest in the brain ammonia level centers around the fact that ammonia is a powerful cerebral irritant; the administration of ammonium salts causes convulsions and it has long been thought that ammonia may play a part in the precipitation of epileptic seizures (3–6). It would appear that the brain ammonia level is closely related to the glutamic acid-glutaminase system, which can remove ammonia by combination with glutamic acid to form glutamine (7). The report that glutamic acid inhibits ammonium chloride convulsions (8) may be significant in this connection, since it is known to reduce the incidence of attacks of petit mal in epileptics (9).

The present paper reports an attempt to obtain reliable values for the ammonia content of rat brain with a method by which the free ammonia can be distinguished from the labile acid amide ammonia. At the same time a study has been made of the effect on the brain ammonia level of convulsant drugs and other factors which might affect it.

EXPERIMENTAL

Ammonia Estimation—Ammonia was estimated by Conway's method (10) of microdiffusion analysis. The acid used was 0.0004 N HCl containing Tashiro's indicator, prepared as described by him (11). The baryta solution (0.0015 N) was delivered from an Agla micrometer syringe (12), which was capable of delivering as little as 0.0001 ml. or considerably less than the error inherent in detecting the indicator change at these dilutions. The stock baryta solution was kept in a rubber-capped bottle of the type used for keeping sterile solutions for injection, which gave a convenient method of refilling the syringe without contamination by CO_2 in the air; the solution was restandardized each day. Preliminary tests with pure crystalline glutamine showed that the rate of liberation of ammonia by hydrolysis was 0.001 mg. of ammonia per mg. of glutamine per hour in contact with 50 per cent-saturated K_2CO_3 at 20° under the experimental conditions of the Conway method. The glutamine in the brain filtrate was determined separately and a correction was calculated on this basis for the brain ammonia figures. The magnitude of the correction was kept low by using a diffusion time of 1 hour. Glutamine is relatively stable at neutral pH at 0°, but it was found to hydrolyze at an appreciable rate, 0.00042 mg. of NH_3 per mg. of glutamine per hour being liberated at a room temperature of 18° in contact with 10 per cent trichloroacetic acid, which was used at approximately this strength in the brain filtrates; the error due to this cause was reduced to a negligible figure by carrying out the ammonia estimations without delay and keeping the solution at 0° with ice. An estimation on a standard solution of ammonium chloride was included in each series.

Glutamine Estimation—Glutamine was estimated by the method of Harris (13), which depends on estimating the ammonia liberated on hydrolysis under standard conditions with 10 per cent trichloroacetic acid at 70° for 75 minutes. Preliminary tests confirmed the observation of Harris that under these conditions a solution containing 50 mg. per cent of pure glutamine was hydrolyzed approximately 100 per cent; under the same conditions asparagine was hydrolyzed 11 per cent, acetamide 69 per cent, and nicotinamide 4 per cent. There was no appreciable liberation of ammonia from D-arginine, guanidine, guanine, adenine, creatine, glutamic acid, or metrazol, but urea liberated 0.002 mg. of NH_3 per mg. under these conditions. Although crystalline glutamine has been isolated from horse brain (14), it was not known to what extent other labile acid amides might be present in the brain. To obtain further evidence on this point, measurements were made of the rates of hydrolysis of the labile acid amides of the brain filtrates and the figures were compared with the rates of hydrolysis of pure glutamine solutions under the same conditions. Four experiments in which a comparison was made of the amount of ammonia liberated from the brain filtrate and from an equivalent amount of pure glutamine on hydrolysis by 50 per cent-saturated K_2CO_3 at 20° are summarized in Table I.

Hydrolysis curves for brain filtrates under acid conditions, carried out as described by Harris (13), also showed good agreement with the curves for pure glutamine, the specific reaction rate k being 7.7×10^{-4} in each case. This gave evidence that the labile acid amide of the brain is mainly, if not wholly, glutamine, and the results are therefore expressed in terms of this substance.

Experimental Animals—In most of the work, young Wistar albino rats weighing 25 to 40 gm. were used. They were killed by being dropped into liquid air, which froze them solid in a few seconds and produced a rapid fixation of the metabolites in the brain. The frozen brain was rapidly removed, crushed to a powder in a cooled steel crusher, and transferred to a previously weighed centrifuge tube containing 4 ml. of 12 per cent trichloroacetic acid at 0°. The mixture was centrifuged and the estimations were carried out on the supernatant solution. An allowance was made in the calculations for the water content of the brain, which was taken as 80 per cent of the weight of the fresh tissue.

In the experiments in which figures for the blood ammonia content were required, larger rats of 200 to 300 gm. were used and were killed by decapitation. The heads were dropped into liquid air and treated in the same way as for the young rats, while blood samples of 0.5 to 1 ml. were pipetted from the carotid artery. The method of killing by decapitation was not entirely satisfactory, for it was found that the stimulus of decapitation was sufficient in itself to cause a rise in the brain ammonia content; but this was the best way in which a blood sample large enough for the ammonia determination could be obtained at the time of fixation of the brain. The rise in the brain ammonia after decapitation continued for about 5 seconds, but by allowing exactly 1 second (timed with a stop-watch) from the moment of decapitation to the commencement of freezing in liquid air, fairly consistent figures could be obtained. In this way changes in the brain ammonia content due to other factors could be demonstrated. Immersion in liquid air must also act as a cerebral stimulus, for it takes several seconds for the brain to freeze right through,[1] but there is evidence that freezing of the intact brain in liquid air is a less violent stimulus than decapitation (15, 16).

TABLE I

Comparison of Ammonia Liberated from Brain Filtrate and from Equivalent Amount of Pure Glutamine

Rat No.	Brain ammonia	Brain glutamine	Time of hydrolysis	Ammonia from brain filtrate	Ammonia from pure glutamine
	mg. per cent	*mg. per cent*	*hrs.*	*mg. per cent*	*mg. per cent*
1	0.59	89	2	0.16	0.18
1	0.59	89	5	0.47	0.45
2	0.54	91	2	0.18	0.18
2	0.59	91	5	0.45	0.46

Results

The values obtained for the ammonia and glutamine content of the brain of normal rats are listed in Table II. The mean figure of 0.28 mg.

---

[1] Richter, D., and Dawson, R. M. C., *Am. J. Physiol.*, in press.

per cent for the ammonia content was considerably lower than the value 2.2 mg. per cent of N previously reported by Bülow and Holmes (1) for the mouse brain. The lower figure obtained in the present work may be attributed partly to the improved method of fixing in liquid air and partly to the correction for ammonia present as glutamine. The mean figure of 79 mg. per cent of glutamine is close to the value of 64 mg. per cent reported by Hamilton (5) for the dog brain.

Emotional Excitement—The recent observation that the brain lactic acid content is increased in emotional excitement (17) suggested that there may also be a liberation of ammonia under these conditions. A series of nine rats was excited by removing their support and allowing them to drop from side to side in a glass beaker for 4 minutes. The method has been described more fully elsewhere.[1] The results, which are listed in Table III, showed a mean ammonia content of 0.22 mg. per cent, which was not significantly different from the normal. There was also no change in the glutamine content.

TABLE II

Ammonia and Glutamine Content of Rat Brain

The rats were killed by immersion in liquid air.

Rat No.	Weight	Ammonia	Glutamine
	gm.	*mg. per cent*	*mg. per cent*
1	35	0.28	62
2	25	0.02	73
3	25	0.34	
4	30	0.14	
5	30	0.35	86
6	35	0.42	81
7	35	0.32	85
8	40	0.25	88
9	30	0.37	
Mean............	32	0.28	79

Picrotoxin Convulsions—Rats killed after convulsions induced by injection of picrotoxin gave a mean value of 0.47 mg. per cent of ammonia, which was 74 per cent above the normal. The difference was statistically significant ($P < 0.05$ when tested by Fisher's t test). There was no significant change in the glutamine level.

Effect of Decapitation—Rats killed by decapitation gave a higher ammonia content than those killed by immersion in liquid air; this was true for young rats of 30 to 40 gm. as well as for adult rats of 200 to 300 gm. (Table IV). The rise in the brain ammonia occurred in the first few seconds

TABLE III

Effect of (a) Emotional Excitement and (b) Picrotoxin Convulsions on Ammonia and Glutamine Content of Rat Brain

The rats weighed 35 to 40 gm. (*a*) The rats were excited for 4 minutes. (*b*) Picrotoxin convulsions were produced by intraperitoneal injection of 0.25 ml. of a solution containing 3 mg. per ml. of picrotoxin in 10 per cent alcohol. All the rats were killed by immersion in liquid air.

Emotional excitement			Picrotoxin convulsions			
Rat No.	Ammonia	Glutamine	Rat No.	Duration of convulsions	Ammonia	Glutamine
	mg. per cent	*mg. per cent*		*min.*	*mg. per cent*	*mg. per cent*
1	0.23	70	10	8	0.57	72
2	0.26	75	11	10	0.44	93
3	0.20		12	5	0.39	68
4	0.14		13	5	0.46	65
5	0.22		14	3	0.52	62
6	0.22		15	3.5	0.60	88
7		78	16	3	0.31	89
8		82				
9	0.27	92				
Mean.....	0.22	79			0.47	77

TABLE IV

Effect of Decapitation on Ammonia Content of Blood and Brain in (a) Young Rats and (b) Adult Rats

Freezing of the heads in liquid air commenced 1 second after decapitation. (*a*) The young rats weighed 30 to 40 gm. (*b*) The adult rats weighed 200 to 300 gm.

Young rats			Adult rats		
Rat No.	Brain ammonia	Blood ammonia	Rat No.	Brain ammonia	Blood ammonia
	mg. per cent	*mg. per cent*		*mg. per cent*	*mg. per cent*
1	0.54	0.35	9		0.19
2		0.23	10		0.19
3	0.35		11	0.57	
4	0.50	0.19	12	0.58	
5	0.60	0.19	13	0.53	
6	0.53		14	0.46	
7		0.32	15	0.33	0.13
8	0.29		16	0.33	
Mean......	0.47	0.26		0.47	0.17

after decapitation. Analyses carried out on a series of six rats examined from 4 to 30 seconds after decapitation gave figures ranging from 0.76 to

1.05 mg. per cent of ammonia with a mean of 0.93 mg. per cent, but by reducing the interval between decapitation and fixing in liquid air lower values were obtained (Table V). With an interval of 1 second between decapitation and freezing in liquid air a mean value of 0.47 mg. per cent of ammonia was obtained and this was adopted as a standard procedure in the experiments with adult rats, which were always killed by decapitation.

TABLE V

Ammonia Content of Rat Brain (a) after Anoxia in Vivo and (b) at Various Times after Decapitation

In series (*a*) young rats of 30 to 40 gm. were rendered anoxic by keeping them for 1 minute in nitrogen containing 5 per cent CO_2; they were killed by decapitation and the heads were frozen after 1 second. The brain ammonia figures should be compared with the mean of 0.47 mg. per cent for normal, decapitated rats. In series (*b*) the heads of rats of 200 to 300 gm. were transferred to liquid air at varying times after decapitation. The heads were kept at 37°.

	Anoxia			Change after decapitation		
Rat No.	Brain ammonia	Blood ammonia	Remarks	Rat No.	Time after decapitation	Brain ammonia
	mg. per cent	*mg. per cent*			*sec.*	*mg. per cent*
1	0.64	0.46	Strong convulsions	6	¾	0.29
2	0.96	0.80	Slight tremors	7	1	0.33
3	0.74		Brief convulsion	8	1½	0.62
4	0.54	0.65	Preconvulsive	9	2	0.86
5	1.18	0.16	"	10	3	0.77
				11	4	0.76
				12	4	1.05
				13	5	0.92
				14	10	0.94
				15	15	0.92
				16	30	1.01
Mean..	0.81	0.52				

Effect of Anoxia—Rats kept for 1 minute in a desiccator containing nitrogen with 5 per cent CO_2 showed a marked increase in the brain ammonia content (Table V); this was observed in animals which had convulsions and also in rats which had not had convulsions but were in the preconvulsive state. It is known that anoxia causes an increase in the blood ammonia level (18) and this may account for a part of the ammonia found in anoxia in the brain; but it is likely that the blood ammonia comes from the brain and other tissues rather than that the tissue ammonia comes from the blood.

Effect of Narcosis—Rats anesthetized for 30 minutes or longer with nembutal showed a marked fall in the brain ammonia content, the mean level for young rats killed by immersion in liquid air being 0.06 mg. per cent of ammonia. The figures, which are listed in Table VI, give evidence that the brain ammonia depends on the duration of the narcosis, for two animals anesthetized for shorter periods of 10 and 17 minutes showed no significant change from the normal. This may be the explanation of the negative findings of Bülow and Holmes (1), who found that narcosis had no effect on the brain ammonia level.

TABLE VI

Effect of Nembutal Narcosis on Ammonia and Glutamine Content of Rat Brain

The brain ammonia figures should be compared with the mean of 0.47 mg. per cent for normal, decapitated rats and 0.28 mg. per cent for normal rats killed by immersion in liquid air.

Rat No.	Weight of rat	Method of killing	Duration of narcosis	Brain ammonia	Brain glutamine
	gm.		*min.*	*mg. per cent*	*mg. per cent*
1	200–300	Decapitation	30	0.15	86
2	200–300	"	30	0.26	84
3	200–300	"	55	0.13	79
4	200–300	"	65	0.23	83
Mean				0.19	83
5	35–40	Liquid air	30	0.05	80
6	35–40	" "	45	0.06	
7	35–40	" "	70	0.15	
8	35–40	" "	80	0.02	
9	35–40	" "	90	0.01	
Mean				0.06	
10	35–40	Liquid air	10	0.29	72
11	35–40	" "	17	0.23	95

Effect of Electrical Stimulation—Electrical stimulation of the brain was carried out with stainless steel electrodes of 0.25 sq. cm. area, which were applied to the scalp 0.5 cm. posterior to the eyes. Contact was obtained by cutting the fur in this region with sharp scissors and applying electrode jelly. The current used was 50 cycles A.C. at 40 volts. Stimulation for 1 to 3 seconds produced a satisfactory convulsion after the usual latent period of about 10 seconds. The procedure was similar to that used in the "electroshock" treatment of psychiatric patients. In the experiments listed in Table VII the rats were sacrificed while undergoing strong convulsions and the figures showed a brain ammonia content above the normal.

Brain Ammonia in Preconvulsive State—The experiments on stimulation by anoxia showed a rise in the brain ammonia level in the preconvulsive state, before the onset of convulsions, which suggested that the rise in brain ammonia might be associated with the increased cerebral irritability rather than with the actual convulsions. The further experiments given in Table VIII confirmed this impression, showing a clear rise in the brain ammonia level in animals sacrificed 1 second after electrical stimulation and therefore several seconds before the onset of convulsions. Estimations of blood ammonia carried out on blood from the carotid arteries of rats killed by decapitation showed a rise in the blood ammonia during electrically induced convulsions; this was to be expected, since it is known that ammonia is liberated in the muscles and enters the blood during severe muscular activity (18). Determinations of blood ammonia on rats in the preconvulsive state gave values which were generally in the normal range, but in a few cases the figures were high; this might be due to the diffusion into the blood of ammonia liberated in the muscles through the tonic mus-

TABLE VII

Ammonia Content of Rat Brain during Convulsions Induced (a) by Electrical Stimulation and (b) by Injection of Ammonium Chloride

The rats were killed by immersion in liquid air or decapitation on the first strong convulsion. The heads of decapitated rats were transferred to liquid air in 1 second. The ammonia content of controls killed by liquid air was 0.27 and killed by decapitation 0.47 mg. per cent. Ammonium chloride convulsions were induced by intraperitoneal injection of 1.2 ml. of 20 per cent NH_4Cl.

Convulsions	Rat No.	Weight of rat	Method of killing	Duration of shock	Brain ammonia	Brain glutamine	Blood ammonia
		gm.		*sec.*	*mg. per cent*	*mg. per cent*	*mg. per cent*
Electric shock	1	35–40	Liquid air	2	0.40	86	
	2	35–40	“ “	3	0.49	76	
	3	200–300	Decapitation	1	0.58	79	
	4	200–300	“	1	0.75	82	
	5	200–300	“	3	0.78		1.42
	6	200–300	“	3	0.94		0.69
Mean, decapitated rats only...........................					0.76		
Ammonium chloride	7	200–300	Decapitation		8.0	70	8.3
	8	200–300	“		9.5	93	9.8
	9	200–300	“		9.2	101	10.0

cular spasm produced by the electrical stimulation. The wide disparity between the ammonia levels in the blood and the brain observed in these and in other experiments gave evidence that the brain ammonia was formed in the brain itself and could not be attributed as a rule to ammonia entering the brain from the blood.

Since the brain ammonia content was raised in the preconvulsive state, it appeared of interest to find out how far the cerebral irritability in this state could be attributed to the toxic action of the free ammonia. It was found that, when ammonium chloride was administered by intraperitoneal injection, convulsions occurred at the time when the brain ammonia level was approximately 9 mg. per cent, or about 10 times the level in the preconvulsive state (Table VII). The brain glutamine content appeared to be raised after ammonium chloride administration, but accurate figures could not be obtained in this series, owing to the large amount of free ammonia present. In contrast to the figures on ammonia, the brain glu-

TABLE VIII

Ammonia Content of Rat Blood and Brain in Preconvulsive State after (a) Picrotoxin Administration and (b) Electrical Stimulation

The rats were killed before the onset of convulsions. Other conditions as in Table VII; picrotoxin dose, 1 ml. of 3 per cent solution.

	Rat No.	Weight of rat	Method of killing	Duration of shock	Brain ammonia	Blood ammonia
		gm.		*sec.*	*mg. per cent*	*mg. per cent*
Picrotoxin	1	200–300	Decapitation		0.65	0.11
	2	200–300	"		0.49	0.42
Electric shock	3	35–40	Liquid air	1	0.40	
	4	35–40	" "	1	0.73	
	5	35–40	" "	1	0.44	
	6	35–40	" "	1	0.41	
Mean, rats killed by liquid air only					0.49	
	7	200–300	Decapitation	2	0.71	0.38
	8	200–300	"	3	0.83	0.13
	9	200–300	"	4	1.04	0.22
	10	200–300	"	3	1.10	0.93
	11	200–300	"	2	0.92	0.32
	12	200–300	"	3	0.92	0.70
	13	200–300	"	2	1.10	0.17
	14	200–300	"	2	0.93	0.20
Mean					0.94	0.38

tamine values showed little variation with the various experimental procedures which were tried.

DISCUSSION

It was found that the ammonia content of the rat brain, analyzed after rapid fixation by freezing in liquid air, was not constant but depended on the state of activity of the brain at the time of fixation. Values obtained on anesthetized animals were significantly lower than those for normal controls, while much higher values were obtained in animals taken during convulsions produced by convulsant drugs or by other methods of stimulation. The brain ammonia content during electrically induced convulsions was more than eight times that found in anesthetized animals. Unless it is believed that these changes occurred during the brief period of freezing with liquid air, it must be concluded that they represent changes occurring *in vivo*. This view is supported by the work of Tashiro (11), who showed that free ammonia is liberated by the isolated frog nerve and that the amount liberated is increased on stimulation. The observations on the frog nerve were confirmed by Winterstein and Hirshberg (19).

The present work showed that the brain contains some source which can liberate up to about 1 mg. per cent of ammonia within 5 seconds upon stimulation. The source of this ammonia is not yet clear, but the brain glutamine content showed little variation, which made it unlikely that glutamine was the source. It might come from the deamination of nucleotides; this has generally been regarded as a relatively slow process (20), but recent work has shown that the simultaneous dephosphorylation and deamination of adenosine diphosphate can proceed with considerable rapidity in muscle preparations (21). It is noteworthy that the liberation of ammonia did not run parallel to the formation of lactic acid in the brain by glycolysis. The brain ammonia and lactic acid are both increased by convulsants and decreased by anesthetics, but there was no liberation of ammonia corresponding to the rise in lactic acid in the brain in emotional excitement.[1] The rapid liberation of ammonia after decapitation came to an end in a few seconds, while the lactic acid formation by postmortem glycolysis continues for a much longer time. Again, the fall in the ammonia content of the brain in anesthesia appeared to follow some time after the decrease in the lactic acid level.

The rise in the brain ammonia content in electrical convulsions was not due to the convulsions themselves, for it could be shown that the liberation of ammonia in the brain occurred in the preconvulsive stage of cerebral irritability before the start of the convulsions. This raised the question of whether the convulsions could be attributed directly to the toxic action of the ammonia. This suggestion was previously made for epileptic convulsions by Riebeling (3) and Brühl (22), who reported that the ammonia content of the cerebrospinal fluid was increased in epileptics after seizures. Richter, Dawson, and Rees[2] were unable to confirm that observation but that did not disprove their hypothesis, for it is not likely that the cerebrospinal fluid would give an accurate reflection of the rapid changes occurring in the brain. The view that epileptic seizures may be attributable to the toxic action of ammonia is attractive, as it can explain a number of the experimentally observed facts; these include (*a*) the precipitation of seizures by anoxia, which has been shown to liberate ammonia in the brain, (*b*) the autocatalytic spread of the discharge, as ammonia liberated at one point stimulates the neighboring regions of the brain, and (*c*) the specific effect of glutamic acid in inhibiting certain types of seizures, in which the rate of detoxication of ammonia might be the limiting factor.

Further experiments designed to test this hypothesis showed that the ammonia liberated in the rat brain on stimulation reached about a tenth of the concentration required to produce convulsions in a normal animal. The figure for the convulsant level was obtained by injecting ammonium chloride and determining the brain ammonia content after the convulsions had started. Under these conditions the ammonia would be fairly evenly distributed throughout the vascular bed and substance of the brain, while it might be expected that the ammonia liberated on stimulation would be formed mainly in the metabolically active regions, where local concentrations higher than the average for the whole brain would be found. The apparent discrepancy between the ammonia level after stimulation and the convulsant level may therefore be less in fact than these experiments would appear to indicate. The present investigation gives evidence that ammonia is liberated in the brain on stimulation and shows that the brain ammonia concentration can approach the toxic range; it suggests that in conditions such as epilepsy, in which the brain is abnormally irritable, the toxic action of ammonia may play a significant part.

SUMMARY

Values are reported for the ammonia and glutamine content of the rat brain. The normal ammonia content, determined by a method which avoided the error due to the decomposition of labile acid amides, was 0.28 mg. per cent and the glutamine content was 79 mg. per cent. A study of the factors affecting the brain ammonia level showed that it was decreased by prolonged nembutal narcosis and markedly increased by direct stimulation of the brain or by procedures which increase cerebral irritability. The ammonia level was increased to 0.47 mg. per cent by picrotoxin ad-

---

[2] Richter, D., Dawson, R. M. C., and Rees, L., *J. Ment. Sc.*, in press.

ministration and 0.81 mg. per cent by anoxia. Electrical stimulation caused a rapid increase in the ammonia level in 1 to 2 seconds and it was also raised by the stimulus of decapitation. The brain ammonia was not affected by emotional excitement and the glutamine content was not significantly affected by any of the factors which were tested. Injection of ammonium chloride in the rat caused convulsions when the brain ammonia level had risen to 9 mg. per cent. The significance of these data in relation to the mechanism of epileptic seizures is discussed.

The authors thank the Medical Research Council for a personal grant to one of us (R. M. C. D.) and for a grant for expenses; they thank the Rockefeller Foundation and the Royal Society for grants for equipment.

BIBLIOGRAPHY

1. Bülow, M., and Holmes, E. G., *Biochem. Z.*, **245**, 459 (1932).
2. Schwarz, H., and Dibold, H., *Biochem. Z.*, **251**, 187 (1932).
3. Riebeling, C., *Klin. Wochschr.*, **10**, 554 (1931); **13**, 1422 (1934).
4. Harris, M. M., *J. Nerv. and Ment. Dis.*, **102**, 466 (1945).
5. Hamilton, P. B., *J. Biol. Chem.*, **158**, 397 (1945).
6. Brain, R., *Proc. Roy. Soc. Med.*, **20**, 861 (1927).
7. Krebs, H. A., *Biochem. J.*, **29**, 1951 (1935).
8. Sapirstein, M. R., *Proc. Soc. Exp. Biol. and Med.*, **52**, 334 (1943).
9. Price, J. C., Waelsch, H., and Putnam, T. J., *J. Am. Med. Assn.*, **122**, 1153 (1943).
10. Conway, E. J., Micro-diffusion analysis and volumetric error, London (1939).
11. Tashiro, S., *Am. J. Physiol.*, **60**, 519 (1922).
12. Trevan, J. W., *Biochem. J.*, **19**, 1111 (1925).
13. Harris, M. M., *J. Clin. Invest.*, **22**, 569 (1943).
14. Ferdman, D. L., Frenkel, S. R., and Silakova, L. I., *Biokhimiya*, **7**, 43 (1943).
15. Avery, B. F., Kerr, S. E., and Ghantus, M., *J. Biol. Chem.*, **110**, 637 (1935).
16. Kerr, S. E., *J. Biol. Chem.*, **110**, 625 (1935).
17. Richter, D., and Dawson, R. M. C., *Nature*, **161**, 205 (1948).
18. Stanojevic, L., Die physiologische Bedeutung und klinische Bedeutung des Blutammoniaks, Leipzig (1938).
19. Winterstein, H., and Hirshberg, E., *Biochem. Z.*, **156**, 138 (1925).
20. Kerr, S. E., *J. Biol. Chem.*, **145**, 647 (1942).
21. Szent-Györgyi, A., Chemistry of muscular contraction, New York, 50 (1947).
22. Brühl, H. H., *Z. Kinderh.*, **59**, 446 (1938).

BIOTIN AND FAT-SOLUBLE MATERIALS WITH BIOTIN ACTIVITY IN THE NUTRITION OF MOSQUITO LARVAE

By WILLIAM TRAGER

(*From the Department of Animal and Plant Pathology, The Rockefeller Institute, Princeton, New Jersey*)

(Received for publication, July 6, 1948)

Recent work has shown that certain microorganisms can under appropriate conditions dispense with biotin if they are supplied with oleic acid or other related materials (1–5). Moreover, an oil from hydrolyzed plasma, as well as a partially purified fraction obtained from it, has been found to have biotin-like activity when injected into young chicks fed a diet high in egg white (2, 6). Oleic acid did not have such activity (6). It seemed of interest to investigate the relationship between biotin and fat-soluble biotin-active substances in the nutrition of an organism entirely different from either bacteria or vertebrates. The yellow fever mosquito *Aedes aegypti* was chosen for this purpose for several reasons. It is among the few insects concerning the nutritional requirements of which considerable information is already available (7). It can easily be maintained in large numbers under laboratory conditions. Its larvae develop rapidly so that definite effects on growth can be discerned within a period of 1 or 2 weeks. The larvae are adapted to life in a liquid medium and can utilize both solid foods and nutrients in solution (8). Finally, *Aedes aegypti* larvae can readily be reared free from microorganisms (9).

This last point cannot be too strongly emphasized. It has become increasingly apparent that some effects of diet may be produced only secondarily via an effect on the synthetic activities of the intestinal bacteria (10–13). Since in nature mosquito larvae feed on microorganisms, nothing whatever can be discovered about their nutritional requirements if living microorganisms are present. Even a slight contamination can permit development in a medium otherwise entirely unsuitable. Hence complete bacterial sterility has been a necessary condition of the experiments. It has at the same time added to the general significance of the results obtained, since in their interpretation there is no need to be concerned with the possible synthetic activities of a microflora either of the intestine or of the environment.

Methods

Colony of Aedes aegypti—This species of mosquito is so adaptable to laboratory conditions that the methods for rearing a stock colony can be varied widely. For the present work the methods were those outlined in a previous paper (14). The schedule of blood meals on a guinea pig was timed in such a manner as to provide twice a week a large batch of eggs known to be less than 24 hours old. These eggs were used to provide bacteria-free larvae for the experiments.

Sterilization of Eggs and Inoculation of Experimental Tubes—These aims were accomplished by the methods previously described (15). Counted numbers (usually three, but sometimes four) of the washed bacteria-free larvae were inoculated into each of the experimental tubes.

Culture Medium and Preparation of Experimental Tubes—Since studies with biotin were contemplated and since biotin is present in most natural foodstuffs, it was necessary to have an essentially synthetic nutrient medium. Previous work had shown that *Aedes aegypti* requires for its larval development thiamine, riboflavin, pantothenic acid, pyridoxine, glutathione, nicotinic acid, choline, and yeast nucleic acid, as well as other unknown factors (7). One of the latter has been found to be folic acid (16), and a requirement for cholesterol has been demonstrated (17). On the basis of these facts it seemed highly probable that a synthetic medium recently developed for the related insect, the fruit fly *Drosophila melanogaster* (18, 19), would also support the growth of *Aedes aegypti*. Such has been found to be the case. Dr. Schultz very kindly supplied me with a description (then not published) of the medium used by him and his co-workers for their studies on the nutrition of *Drosophila*. This medium has been modified in the following two ways. Firstly, whole vitamin-free casein has been used instead of casein hydrolysate plus tryptophan, since the latter supported only very slow growth in a medium in which the former supported growth at a nearly normal rate. Secondly, pyridoxamine as well as pyridoxine and niacin as well as niacinamide were included in the medium. The final biotin-free medium which was added to vitamin-free casein (General Biochemicals, Inc.) had the composition shown in Table I. This medium may be prepared by any of several methods. The following has been routinely used for the present investigation. The cholesterol was prepared as a separate suspension. 300 mg. of cholesterol were dissolved in 20 ml. of 95 per cent ethyl alcohol. This solution was added in 1 ml. amounts to tubes containing 9 ml. of distilled water, giving a uniform white suspension. The tubes were autoclaved 20 minutes at 15 pounds to sterilize them and to drive off the alcohol, leaving a volume of approximately 8 to 9 ml. in each. They were stored in a refrigerator. For use, 0.1 ml. of suspension was added aseptically to each tube which was to contain 6 ml. of final medium. Some indication was obtained that the cholesterol suspension began to lose its effectiveness after more than 2 months of storage in the refrigerator.

The remainder of the medium was prepared at twice its final concentration. The required amount of cystine was dissolved in a minimal volume of hot 6 N hydrochloric acid and was added to a suspension of the nucleic acid in a volume of water half that of the desired amount of double strength medium. 2 N sodium hydroxide was added to dissolve the nucleic acid and bring the pH to 6.8 to 6.9. The sucrose was then dissolved. The salts and the growth factors were added from concentrated stock solutions kept under toluene in the refrigerator, except for the calcium chloride, niacinamide, glutathione, choline, and inositol, which were added from freshly prepared more concentrated solutions. The pH was readjusted if necessary to 6.8, to 6.9, and the medium was diluted to the appropriate volume. It was sterilized, in batches of 200 ml., by filtration through a Selas 03 porcelain filter, since preliminary experiments had shown that growth on this medium sterilized by autoclaving was inferior to that on the same medium sterilized by filtration. The sterile medium was stored in a refrigerator and used usually within less than 1 week after preparation.

TABLE I

Composition of Synthetic Medium for Aedes aegypti Exclusive of Biotin and Casein

Material	Concentration	Material	Concentration
	mg. per ml.		*γ per ml.*
KH_2PO_4	0.60	Pyridoxamine dihydrochloride	0.02
K_2HPO_4	0.60	Pyridoxine hydrochloride	4.0
$FeSO_4 \cdot 7H_2O$	0.012	Riboflavin	2.0
$MnSO_4 \cdot 4H_2O$	0.012	Thiamine hydrochloride	2.0
NaCl	0.012	Niacin	2.0
$MgSO_4 \cdot 7H_2O$	0.20	Calcium pantothenate	6.0
$CaCl_2$	0.012	Pteroylglutamic acid	0.6
Ribose nucleic acid	1.0	*p*-Aminobenzoic acid	2.0
L-Cystine	0.2	Niacinamide	10.0
Cholesterol	0.03	Glutathione	10.0
i-Inositol	0.04	Choline chloride	20.0
Sucrose	2.0		

It was found convenient to supply the casein in solid form (8) rather than in solution. In this way the larvae, which are bottom feeders, were provided with some particulate food at the bottom of the tube. A small spatula which delivered 30 to 40 mg. of the dry powdered casein was used to measure this material into each tube of a group of 18 × 160 mm. test-tubes. The tubes were plugged with non-absorbent cotton and were sterilized by dry heat in an automatic electric oven at 160–170° for 2 hours. This treatment slightly darkened the surface of the casein but did not otherwise affect it.

For any one experiment, all the tubes were prepared at the same time and all received the same batch of double strength medium and of cholesterol. Duplicate tubes were usually prepared for each factor being tested, although sometimes three or even five tubes of each were used. Each experiment included control tubes to which no biotin was added, as well as tubes with various known concentrations of biotin. The biotin was added from an autoclaved solution standardized by microbiological assay and stored in the refrigerator. Water-soluble materials to be tested for biotin activity were similarly added from autoclaved stock solutions. Materials insoluble in water, such as oleic acid and the biotin-active oil from hydrolyzed horse plasma (hereafter designated FSF), were emulsified by means of ethyl alcohol. The emulsions were prepared as follows. To 0.95 ml. of warm 95 per cent alcohol in a sterile tube was added 0.05 ml. of the oil, previously autoclaved. 9 ml. of sterile distilled water were then run in with shaking. A milky emulsion formed with some free oil droplets which floated to the surface and were avoided in making the subsequent dilutions. Usually 1 ml. of emulsion was diluted in 9 ml. of sterile water, and the dilute uniform emulsion was added to the experimental tubes in amounts up to 1 ml. The maximal concentration of alcohol was hence only about 0.15 per cent, which was not found to have any effect on growth in the synthetic medium supplemented with biotin. The concentration of active material in the final dilute emulsions was determined by microbiological assay.

In the preparation of an experimental series, the appropriate quantity of sterile distilled water (enough to give a final volume of 6 ml.) was first pipetted into each sterile tube containing casein. This was followed by 3 ml. of the double strength medium and 0.1 ml. of the cholesterol suspension. The biotin or other supplements were then added and the tubes were inoculated with the washed first instar larvae. The usual precautions to insure sterility were observed. The tubes were kept in an incubator at 28–29°, except for a brief period each day when they were removed for examination.

Microbiological Assays—Biotin and the biotin activity of lipide materials were assayed with *Lactobacillus casei* with the method of Landy and Dicken (20) slightly modified (2).

Sterility Tests—Since most of the media used were clear, nearly colorless liquids, contamination could often be detected by the appearance of cloudiness. All tubes after about 7 days of incubation were submitted to a routine sterility test. This consisted of streaking a loopful of material from each tube onto a plate of dextrose nutrient agar. All tubes which showed some new effect, or the results of which would be especially significant, were later submitted to additional sterility tests in nutrient broth, yeast extract agar, and cooked meat medium. Contaminations were detected in about 1 per cent of the tubes. As soon as an experimental tube showed evidence of being contaminated it was discarded and the results previously obtained with it were not used.

Quantitative Expression of Developmental Rate—The larvae of *Aedes aegypti* undergo three molts between hatching from the egg and pupation. Hence there are four larval instars. These provide the basis for a simple method for following the rate of development. One needs only to examine each tube daily and to note the number of larvae in each instar. Although the larvae grow in body length between molts, their head and anal siphon show a discontinuous growth with a marked increase in size immediately following each molt. This fact makes it possible to recognize at a glance whether a larva is in the first, second, third, or fourth instar (see Fig. 1). This method of following growth was used in the early work on the nutrition of mosquito larvae (15). At that time a growth index was described which was obtained from the expression $N \times (1/T)$, in which N is the percentage of larvae reaching the fourth instar within a chosen period of time and T is the average time in days required by them to do so. The choice of the time period does not materially affect the relative results. A longer period permits a larger percentage to reach the fourth instar in the less

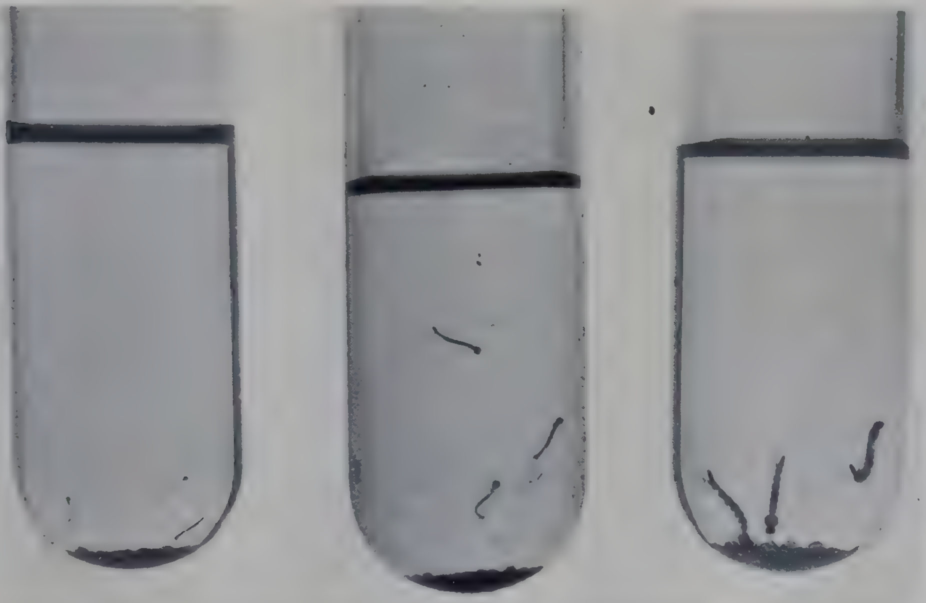


FIG. 1. *Aedes aegypti* larvae 7 days after their inoculation as first instar larvae into (from left to right) (*a*) the basal medium without added biotin (all in the second instar); (*b*) the basal medium plus 4.2 mγ of biotin per ml. (all in the third instar); (*c*) the basal medium plus 33.3 mγ of biotin per ml. (all in the fourth instar). Photograph by J. A. Carlile.

nearly adequate media, but, since they take longer to do so, the final result is but little changed. For the present work a time of 14 days was chosen, since in this period very few or none of the larvae in the medium without added biotin reached the fourth instar, whereas in the presence of sufficient biotin all reached the fourth instar. Therefore all tubes were examined daily for the first 14 days in order to secure the data for the calculation of $N \times (1/T)$. They were then examined every other day for an additional 14 days in order to follow the emergence of adults and to check on the possibility of an unusual delayed growth.

TABLE II

Effect of Addition of Different Concentrations of Biotin to Synthetic Medium on Growth and Development of Aedes aegypti

Concentration of added biotin	No. of larvae from group of 6, reaching within 14 days		Adults		Average time to			$N \times \frac{1}{T}$*
	3rd instar	4th instar	Female	Male	3rd instar	4th instar	Adult	
mγ per ml.					*days*	*days*	*days*	
0	5	0	0	0	10.2			0
6.7	6	6	1	2	5.2	10.3	20	9.7
10.0	6	6		3	4.3	8.0	18	12.5
13.3	6	6	1	1	3.3	8.2	14	12.2
16.7	6	6	2	4	3.5	6.3	14	15.9
33.3	6	5	2	3	3.5	6.4	14	13.0
66.7	6	6	3	3	3.3	4.8	10	20.8
166.7	5	5		3	4.4	7.0	11	11.9

* In this and the following tables $N \times (1/T)$ is an expression of the over-all rate of larval development in which N is the percentage of larvae reaching the fourth instar within 14 days and T is the average time in days required by these larvae to reach the fourth instar. Under optimal conditions (15) all the larvae reach the fourth instar on the 4th day, so that the maximal value for $N \times (1/T)$ is 25. Unless otherwise stated, each value is based on the growth of six larvae, three each in two tubes.

Results

Biotin and Pimelic Acid—In the synthetic medium in the absence of added biotin few or none of the larvae attained the fourth instar within 2 weeks and none ever pupated. Biotin concentrations as low as 0.6 mγ per ml. had a distinct effect on larval growth, and the value of $N \times (1/T)$ became progressively higher with increasing concentrations of biotin, until it reached a maximum of 20 in the presence of 66.7 mγ per ml. (Tables II and III; Fig. 1). The poorer growth obtained with a still higher concentration of biotin (166.7 mγ per ml., Table II) suggests the possibility of a toxic effect of too much biotin, which has been previously reported for the flour beetle (21). Too few trials have been made at this high level to permit a definite statement. While a biotin concentration of 50 mγ per ml. or somewhat more was required to give the optimal growth obtainable under the conditions of these experiments, in the presence of only about 20 mγ per ml. most of the larvae metamorphosed into adult mosquitoes, and even with as little as 5 to 6 mγ per ml. a few succeeded in reaching the adult stage. With lower concentrations of biotin the larvae failed to pupate.

TABLE III

Replacement of Biotin in Larval Growth of Aedes aegypti by Oleic Acid, FSF, and Two Partially Purified Fractions from FSF, Tween 80 and Tween 40

Supplement	Concentration per ml.		$N \times \frac{1}{T}$*
	Actual mγ	As mγ biotin activity for *L. casei*	
None	0	0	0.8
Biotin	0.6	0.6	2.5
	2.1	2.1	1.9
	6.2	6.2	6.9
	21.0	21.0	14.3
Oleic acid	2,400	0.02	3.6
	4,800	0.04	4.2
	7,200	0.06	4.6
FSF	3,500	0.007	4.0
	7,000	0.014	6.3
	10,500	0.021	6.4
Cold alcohol-soluble fraction from FSF	3,750	0.015	1.5
	5,000	0.02	3.5
	6,250	0.025	3.3
Ammoniacal eluate from aluminum oxide adsorption of FSF	1,200	0.01	2.6
	1,800	0.015	8.5
	2,400	0.02	4.4
Tween 80	67,000	0.067	4.3
	134,000	0.134	4.7
	333,000	0.333	2.5
" 40	50,000	0.001	0
	100,000	0.002	0
	200,000	0.004	4.3
	400,000	0.008	6.1

Table II shows the adequacy of the single number $N \times (1/T)$ as an index of larval development. A similar figure could be calculated for the third instar and the same relative values would be obtained in most instances. All larvae reached the second instar on the 2nd or 3rd day regardless of the presence of added biotin or other supplements unless inhibitory substances were present, in which case they might remain in the first instar for a week or longer before dying. It is also evident from Table II that the value of $N \times (1/T)$ gives some indication of the extent of metamorphosis to the adult stage. In general, with $N \times (1/T)$ values of about 15 most of the larvae became adults in about 2 weeks. With $N \times (1/T)$ values around 20, which is the highest yet observed in the synthetic medium and is close to the maximal possible value of 25, all or most of the larvae became adults in 10 to 12 days. When $N \times (1/T)$ was 10 or less, only a few or none of the larvae succeeded in reaching the adult stage within the observation period of 4 weeks. A longer period of observation would have been of little value, for larvae which reached the fourth instar but failed to pupate within 4 weeks usually began to show signs of weakness at about that time.

---

* The figures for no supplement, biotin, oleic acid, and FSF are each based on nine larvae, while those for the other supplements are each based on six larvae.

Pimelic acid can apparently replace biotin in the nutrition of the flour beetle *Tribolium confusum* (22), serving presumably as a precursor of biotin (23–25). Since *Tribolium* is the only multicellular animal which has been reported as capable of converting pimelic acid to biotin, an attempt was made to discover whether *Aedes aegypti* could also do this. The results of several experiments have been negative. For example, the same experiment in which the results with biotin are given in Table II included the testing of two different samples of pimelic acid, each at concentrations of 3.3, 6.7, 10.0, 13.3, 16.7, 33.3, 66.7, and 166.7 mγ per ml. $N \times (1/T)$ was 0, except in the highest concentration of one sample, for which it was 1.5. There was no significant acceleration of growth over that observed in the tubes without any supplement. It must be concluded that either *Tribolium* has greater synthetic powers than *Aedes*, or its ability to utilize pimelic acid depended on the synthetic activities of microorganisms present in the intestine of the beetle or in the diet, since the experiments with *Tribolium* were not carried on under aseptic conditions.

Replacement of Biotin by Oleic Acid, FSF, and Related Substances—Both oleic acid and the oil from hydrolyzed plasma (FSF), when added in place of biotin, had a definite effect on larval growth (Table III). It was important to choose an appropriate range of concentrations. Growth stimulation failed to occur if the concentration was either too low or too high. In the latter case, the materials probably exerted a toxic effect, for with the somewhat higher concentrations most of the larvae remained in the first instar and with still higher concentrations they were killed within 1 day. The range of effectiveness of oleic acid was smaller than that of FSF and the maximal value of $N \times (1/T)$ obtained in all the experiments in which the two materials were compared was smaller for oleic acid than for FSF. With FSF itself and some active fractions derived from it, values for $N \times (1/T)$ of up to 10 to 11 have been frequently obtained, but the highest ever observed for oleic acid was 6.8, and this in only one experiment. Both oleic acid and FSF, if measured in terms of their microbiological biotin activity, would seem to be much more active for mosquito larvae than biotin itself (see, for example, Table III). They are of course much less active if measured in terms of their actual concentration.

Several partially purified fractions which were obtained from FSF (6) and which had biotin activity for *Lactobacillus casei* also had biotin activity for *Aedes aegypti* larvae. The data obtained with two such fractions are given in Table III. The results with the ammoniacal eluate are of special interest, since this material has also been found to have biotin-like activity when injected into chicks on an egg white diet and to have almost as high a specific activity for *Lactobacillus casei* as has oleic acid (6).

A variety of synthetic detergents, chiefly non-ionic esters of fatty acids, has been found to function as growth factors for certain bacteria (26, 5) and to be capable, like oleic acid and FSF, of replacing biotin in the nutrition of lactic acid bacteria (4). Two of these materials, Tween 80 (a polyoxyethylene derivative of sorbitan monooleate) and Tween 40 (a similar monopalmitate), have been tested and found to have some activity for the growth of *Aedes aegypti* when added to a biotin-free medium (Table III).

As might have been expected, lecithin can replace oleic acid for those bacteria for which the latter substance has a growth-promoting effect (5). It can similarly replace biotin in the growth of *Aedes aegypti* (Table IV). At concentrations higher than those shown in Table IV animal lecithin became progressively more and more inhibitory, although even at very high concentrations it did not kill the larvae, which lived for a week or 2 in the first or second instar. Like oleic acid and FSF, lecithin, when used in place of biotin, had a much greater effect on the growth of *A. aegypti* larvae than would be expected from its biotin activity for *Lactobacillus casei*. Thus a concentration (15 γ per ml.) with a biotin activity for *L. casei* of only 0.023 mγ per ml. gave as high a value for $N \times (1/T)$ as was usually obtained with biotin concentrations of 4 to 6 mγ per ml. This same fact is illustrated by the activity of lecithin when added as a supplement to a low concentration of biotin (Table IV). The addition of 5 γ per ml. of lecithin, which theoretically increased the biotin activity for *L. casei* from only 4.2 to 4.208 mγ per ml., raised the $N \times (1/T)$ value from 7 to 15 and permitted the emergence of some adults. The higher concentrations of lecithin, when added to this low concentration of biotin, had less effect, which was the reverse of the situation with lecithin alone. Other experiments have similarly indicated the possibility of an optimal ratio between biotin and lecithin but more work will have to be done to establish this. It is interesting that lecithin was very early found to affect the growth of *Drosophila* under sterile conditions (27).

In the many experimental tubes with different concentrations of oleic acid and FSF, pupation has never occurred. Among the comparatively fewer tubes containing lecithin at a suitable concentration a viable pupa has been noted in two different experiments. Both pupae died without completing transformation to the adult stage. Since complete tests of the sterility of the tubes did not reveal any contamination, it seems likely that lecithin or some impurity in it is a more suitable source of lipide to replace biotin than either oleic acid or FSF.

TABLE IV

Effect of Lecithin in Absence of Biotin and in Presence of Low Concentration of Biotin on Growth of Aedes aegypti

Supplement	Concentration	$N \times \frac{1}{T}$	Adults	
			Female	Male
	mγ per ml.			
None	0	2.6	0	0
Lecithin*	5,000	5.2	0	0
	15,000	9.0	0	0‡
	30,000	12.1	0	0
Biotin	4.2	7.2	0	0
Biotin† + lecithin	5,000	15.0	3	0
	15,000	13.4	0	1
	30,000	8.3	2	1

* The lecithin used had a biotin activity for *Lactobacillus casei* of 1.5 mγ per mg. or 0.0075 mγ per 5000 mγ.

† Each tube contained biotin at 4.2 mγ per ml. + the indicated concentration of lecithin.

‡ One larva pupated on the 26th day but failed to emerge as an adult.

DISCUSSION

Aedes aegypti is now the second insect, and also only the second multicellular animal, to be reared from egg to adult in the absence of microorganisms and on a diet of essentially known composition, *Drosophila melanogaster* having been the first (18). For *Aedes* as for *Drosophila*, the synthetic medium is not quite adequate. More rapid growth and more vigorous adults are regularly observed in sterile media containing certain natural foodstuffs. The unknown factor for both *Drosophila* (18) and *Aedes* (17) is present in the water-insoluble fraction of yeast, and that for *Drosophila* at least is also present in water-soluble form in yeast autolysate (28).

The growth of *Aedes aegypti* in the complete synthetic medium is nevertheless sufficiently good to permit the evaluation of the rôle of individual metabolites in the growth of this insect. It has been possible to show that biotin is essential for the development of *A. aegypti*, as it is for other insects (21, 29), and to determine quantitatively the requirements of this insect for biotin. For *Tribolium confusum* the optimal concentration of biotin was found to be 100 mγ per gm. of diet, with as little as 6 mγ per gm. producing detectable effects (21). For *A. aegypti* the optimal concentration lies around 50 mγ per ml. of liquid medium, with detectable effects at concentrations down to 0.6 mγ per ml.

In the presence of casein, sucrose, nucleic acid, and all the known B vitamins, including biotin, *Aedes aegypti* does not require any fat-soluble growth factor other than cholesterol. In this respect it resembles *Drosophila* and several species of beetles (7) and differs from the moths of the genus *Ephestia*, which require linoleic acid even on a diet containing yeast (30). The growth-stimulating effects, in a medium essentially free from biotin, of oleic acid, FSF, lecithin, and related compounds on the larvae of *A. aegypti* may be taken to indicate that biotin functions in the synthesis of such fatty compounds. If suitable lipides are present in the diet, biotin may be largely dispensed with. Such an explanation has been suggested for the ability of some lactic acid bacteria to dispense with biotin in the presence of oleic acid (5) and has been discussed in relation to the biotin-like activity in chicks of the neutral oil from plasma (6). The best larval growth of *A. aegypti* which has been obtained with oleic acid in place of biotin has not been as good as that supported by the lowest concentration of biotin which sufficed for the metamorphosis of some of the larvae to the adult stage. However, with FSF and fractions derived from it, and with lecithin, at appropriate concentrations the rate of larval development has been about as great as the minimum which, in the presence of low concentrations of biotin, was compatible with the occasional emergence of adults. And yet no metamorphosis occurred, with the exception of the two pupae observed in tubes containing lecithin. It may be merely that the lipides, because of their toxic effects, cannot be provided in the nutrient medium at a concentration high enough to carry the larvae through into metamorphosis. Or it may be that biotin is essential also for the synthesis of some particular lipide or other type of compound which has not been provided in the experiments reported here and which is required for metamorphosis but not for larval growth.

SUMMARY

The yellow fever mosquito *Aedes aegypti* has been grown from the egg to the adult stage in a medium free from microorganisms and of essentially

known composition. If biotin was omitted from the medium, larval growth was very slow and metamorphosis to the adult stage did not occur. Optimal growth on the synthetic medium was slightly inferior to that observed in liver extract plus heat-killed yeast and was obtained in the presence of about 50 mγ of biotin per ml. of medium. With lower concentrations of biotin growth was progressively slower, and with less than about 5 mγ per ml. metamorphosis to the adult stage did not take place, although clearly recognizable effects on larval growth were produced by concentrations as low as 0.6 mγ per ml. Biotin could not be replaced by pimelic acid.

Relatively low concentrations of oleic acid, an oil from hydrolyzed plasma (FSF), lecithin, and related compounds, when used in place of biotin, supported larval growth as good as that obtained with the lower effective concentrations of biotin.

For the mosquito *Aedes aegypti*, as well as for some bacteria and for chicks, certain lipide compounds can at least partially replace biotin, suggesting that biotin must be of general importance in the synthesis of these lipides.

It is a pleasure to acknowledge the faithful and efficient technical assistance of Miss Marion Orcutt and Mrs. Anne Baldino.

BIBLIOGRAPHY

1. Williams, V. R., and Fieger, E. A., *J. Biol. Chem.*, **166**, 335 (1946).
2. Trager, W., *Proc. Soc. Exp. Biol. and Med.*, **64**, 129 (1947).
3. Axelrod, A. E., Hofmann, K., and Daubert, B. F., *J. Biol. Chem.*, **169**, 761 (1947).
4. Williams, V. R., and Fieger, E. A., *J. Biol. Chem.*, **170**, 399 (1947).
5. Williams, W. L., Broquist, H. P., and Snell, E. E., *J. Biol. Chem.*, **170**, 619 (1947).
6. Trager, W., *J. Biol. Chem.*, **176**, 133 (1948).
7. Trager, W., *Biol. Rev., Cambridge Phil. Soc.*, **22**, 148 (1947).
8. Trager, W., *Biol. Bull.*, **71**, 343 (1936).
9. Trager, W., *Am. J. Hyg.*, **22**, 18, 475 (1935).
10. Almquist, H. J., *Physiol. Rev.*, **21**, 194 (1941).
11. Williams, R. J., Annual review of biochemistry, Stanford University, **12**, 305 (1943).
12. Cravens, W. W., and Couch, J. R., *Federation Proc.*, **6**, 404 (1947).
13. Gall, L. S., Illingworth, B. A., Cowgill, G. R., and Fenton, P. F., *J. Nutr.*, **35**, 27 (1948).
14. Trager, W., *J. Parasitol.*, **28**, 457 (1942).
15. Trager, W., *J. Exp. Biol.*, **14**, 240 (1937).
16. Golberg, L., de Meillon, B., and Lavoipierre, M., *J. Exp. Biol.*, **21**, 90 (1945).
17. Golberg, L., and de Meillon, B., *Nature*, **160**, 582 (1947).
18. Schultz, J., St. Lawrence, P., and Newmeyer, D., *Anat. Rec.*, **96**, 44 (1946).
19. Villee, C. A., and Bissell, H. B., *J. Biol. Chem.*, **172**, 59 (1948).
20. Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, **27**, 1086 (1942).
21. Fraenkel, G., and Blewett, M., *Biochem. J.*, **37**, 686 (1943).

22. Rosenthal, H., and Grob, C. A., *Z. Vitaminforsch.*, **17**, 27 (1946).
23. Mueller, J. H., *J. Biol. Chem.*, **119**, 121 (1937).
24. du Vigneaud, V., Dittmer, K., Hague, E., and Long, B., *Science*, **96**, 186 (1942).
25. Eakin, R. E., and Eakin, E. A., *Science*, **96**, 187 (1942).
26. Dubos, R. J., *J. Exp. Med.*, **85**, 9 (1947).
27. Delcourt, A., and Guyénot, E., *Bull. sc. France et Belgique*, **45**, 249 (1911).
28. Begg, M., and Robertson, F. W., *Nature*, **161**, 769 (1948).
29. Sarma, P. S., *Indian J. Med. Res.*, **32**, 149 (1944).
30. Fraenkel, G., and Blewett, M., *Biochem. J.*, **41**, p. xviii (1947).

TISSUE PROTEINS AND CARCINOGENESIS

I. THE EFFECT OF CARCINOGENIC AZO DYES ON LIVER PROTEINS

BY A. CLARK GRIFFIN, WILLIAM N. NYE, LAFAYETTE NODA,
AND J. MURRAY LUCK

(From the Department of Chemistry, Stanford University, California)

(Received for publication, August 11, 1948)

Certain constituents of the liver undergo changes which accompany the feeding of the carcinogenic azo dyes. Hoch-Ligeti (1) noted that the activity of the enzyme, succinoxidase, is reduced in livers of rats fed *p*-dimethylaminoazobenzene. Liver tumors induced by this carcinogen have lower cytochrome oxidase and succinoxidase activities than normal liver (2). The azo dyes also lower liver riboflavin in proportion to the carcinogenic activity of the dye (3–5). Recently Miller and Miller (6) demonstrated a firm binding of azo dye to liver proteins before actual tumor formation. Other workers (7, 8) have observed that hepatomas induced by the azo dyes contain more desoxyribonucleic acid than normal tissues, while the ribonucleic acid remains normal or is slightly reduced. Of equal importance are the observations of Price, Miller, and Miller (9) and Masayama and Yokoyama (10) that the precancerous livers of rats fed diets containing *p*-dimethylaminoazobenzene exhibit increased levels of desoxyribonucleic acid. Opie (11) found definite changes in the cytoplasmic ribonucleic acids of liver cells when tumors were produced by the feeding of *p*-dimethylaminoazobenzene.

In the present study livers of rats were fractionated in order to isolate the desoxyribonucleoproteins, ribonucleoproteins, albumins, and globulins. Analyses were made for these components as well as for nitrogen, phosphorus, and riboflavin in the various fractions obtained. The fractionation and analytical studies were carried out on normal rat liver, precancerous liver, and hepatomas resulting from the feeding of diets containing *m'*-methyl-*p*-dimethylaminoazobenzene; this is one of the most active carcinogens of the azo dye group (12).

Methods

Male albino rats,[1] weighing approximately 200 to 250 gm., were fed diets containing 0.06 per cent *m'*-methyl-*p*-dimethylaminoazobenzene (*m'*Me-DAB) *ad libitum*, for periods up to 10 weeks. The purified basal diet was similar to that used by the Wisconsin group (13), and contained casein 18 per cent, glucose monohydrate 73 per cent, corn oil (Mazola) 5 per cent, salts mixture[2] 4 per cent. To each kilo of the diet so prepared were added thiamine hydrochloride 3.0 mg., riboflavin 2.0 mg., calcium pantothenate 7.0 mg., pyridoxine hydrochloride 2.5 mg., and choline 0.5 gm. Each animal was given 2 to 3 drops of halibut liver oil by a dropper at 2 week intervals.

---

[1] Holtzman, Sprague-Dawley strain.

The animals were anesthetized with ether and the livers perfused *in situ* with cold 0.14 M NaCl. The livers from five to six rats usually constituted one group for the fractionation and analytical procedures. The excised livers were rapidly weighed and samples removed for moisture determinations. To the remaining liver were added 3.5 parts by weight of neutral 0.4 M NaCl and the mixture was homogenized for 2 minutes in a Waring blendor. After 10 to 15 minutes of stirring, this homogenate was centrifuged for 15 minutes at 4000 R.P.M. (International centrifuge No. 2, conical head). The supernatant fluid was removed and the residue again extracted with a volume of 0.4 M NaCl equal to that of the above supernatant fluid. Following this extraction the remaining residue was stirred with 1.5 volumes of 1.0 M NaCl and centrifuged for 1 hour at 4000 R.P.M. as above. The total homogenate, the first and second extracts with 0.4 M NaCl, the 1.0 M NaCl extract, and the final residue were all analyzed for desoxyribonucleoproteins (DNP) and ribonucleoproteins (RNP) by the hot trichloroacetic acid method of Schneider (14), for nitrogen (15), phosphorus (16), and riboflavin (17, 18).

Albumin and globulin values were determined by analysis of the initial 0.4 M NaCl extract. The extract was reduced to pH 5.0, allowed to stand for 1 hour, and was then centrifuged for 1 hour at 4000 R.P.M. The supernatant fluid containing the globulins and albumin was dialyzed overnight with running distilled water in a rocking dialyzer. The precipitated globulin was centrifuged off and combined with the globulin fraction obtained by precipitation in 2.3 M ammonium sulfate at pH 7.0. The further addition of solid ammonium sulfate to 4.6 M and adjustment of the pH to 6.5 precipitated the albumin fraction. Analysis for the fractions designated as globulin and albumin was made by a modification of the biuret method of Robinson and Hogden (19) by using the linear relationship of optical density to concentration as obtained from crystalline bovine serum albumin. The entire fractionation procedure was carried out in a room maintained at 1°. Special precautions were taken to maintain this temperature during homogenization, centrifugation, etc.

---

[2] Salts mixture (parts by weight): NaCl 1470, $Ca_3(PO_4)_2$ 2086, $MgSO_4 \cdot 7H_2O$ 588, KCl 1680, $CaCO_3$ 2940, $FePO_4 \cdot 4H_2O$ 206, KH_2PO_4 4340, $MgCO_3$ 672, $MnSO_4 \cdot H_2O$ 3.2, $K_2Al_2(SO_4)_2$, $12H_2O$ 1.2, $CuSO_4 \cdot 5H_2O$ 5.4, NaF 7.4, and KI 1.2.

Results

Fractionation of Normal Rat Liver—The initial fractionations were carried out by successive extractions of homogenized rat liver with 0.14 M NaCl as described by Mirsky and Pollister (20). It was difficult, however, to remove more than 40 per cent of the total RNP with this concentration of NaCl. Various combinations of extractions were carried out to improve the method. Three successive extractions with 0.4 M, 0.28 M, and 0.14 M NaCl removed 85 per cent of the total RNP and 8 per cent of the total DNP. It was noted, however, that most of the RNP appeared in the initial 0.4 M NaCl extract. When the extractions were carried out with NaCl solutions of a higher concentration than 0.4 M (*i.e.*, 0.5 to 0.6), slightly more RNP was removed but increasing amounts of the DNP were extracted. The viscosity of the homogenate increased at salt concentrations above 0.4 M, and at 0.6 M the mixture was extremely viscous, indicating solvation of the DNP. From histological data it was apparent that the nuclei, which contain most of the DNP, remained relatively intact up to NaCl concentrations of 0.4 M. These observations, along with analytical data, prompted the use of 0.4 M NaCl for the initial extractions. The complete method has already been described. The values obtained for the different components in the various fractions are presented in Table I.

TABLE I

Liver Fractionation

Mg. per 100 gm. of fresh liver.*

Liver fraction	Nitrogen		Phosphorus		DNP†		RNP‡		Riboflavin	
	mg. per cent	*per cent of total*	*mg. per cent*	*per cent of total*	*mg. per cent*	*per cent of total*	*mg. per cent*	*per cent of total*	*γ per cent*	*per cent of total*
Total homogenate	2520		280		266		150		2466	
1st extraction, 0.4 M NaCl	1400	56	164	58	19	7	98	65	1007	41
2nd " 0.4 M NaCl	258	10	27	10	8	3	18	12	182	7
Extraction, 1.0 M NaCl	250	10	39	14	176	66	15	10	206	8
Final residue	602	24	52	18	61	23	20	13	1134	46

* The values expressed are the averages of six fractionations, five or more rats per group. Rats maintained on the basal purified diet 4 weeks. Liver moisture, 74 to 76 per cent. Average daily food intake, 18 gm. per rat; average liver weight, 10.0 gm.

† Desoxyribonucleoprotein as desoxyribonucleic acid.

‡ Ribonucleoprotein as ribose.

The initial extraction with 0.4 M NaCl removed over 50 per cent of the total nitrogen and phosphorus, 41 per cent of the total riboflavin, 7 per cent of the DNP, and 65 per cent of the RNP. All of the soluble globulin and albumin appeared in this extract. Subsequent extraction with 1.0 M NaCl resulted in a fraction containing 66 per cent or more of the liver DNP, 14 per cent of the total phosphorus, and approximately 10 per cent of the total RNP, riboflavin, and nitrogen. The final residue contained 23 per cent of the DNP. This can be removed, if desired, by exhaustive extraction with 1 M NaCl. Riboflavin also appeared to concentrate in this fraction, presumably in combination with the sodium chloride-insoluble protein fraction.

Table II

Effect of Carcinogenic Azo Dye on Liver Proteins

Purified basal diet + 0.06 per cent *m'*-methyl-*p*-dimethylaminoazobenzene.

Component	Control basal diet	Time rats were fed diet containing azo dye				Hepatomas
		2 wks.	4 wks.	6 wks.	8 wks.	
Nitrogen, *mg.* %	2520	2470	2470	2500	2465	2040
Phosphorus, *mg.* %	280	316	316	308	326	272
Desoxyribonucleoprotein, as desoxyribonucleic acid, *mg.* %	266	396	440	490	506	756
Ribonucleoprotein, as ribose, *mg.* %	150	122	146	140	126	139
Riboflavin, γ %	2466	1967	1740	1395	1278	
Albumin, %	1.3	1.1	1.2	1.1	1.1	
Globulin, %	1.3	2.1	2.0	2.2	2.2	
Non-protein nitrogen, *mg.* %	180	181	213	205	230	
" phosphorus, *mg.* %	81	98	104	108	108	

The values are expressed on a fresh liver basis and are averages of three or more groups per period, four to six rats per group. Cirrhosis was evident after 4 weeks and at 8 weeks most of the livers were extremely cirrhotic. Liver moisture, 75 to 78 per cent. Food intake while on diet containing azo dye, 13.5 to 15 gm. per rat per day. Average liver weight, 9.7 gm. per rat (range 7.2 to 13.5).

It is apparent that this procedure provides only crude fractions. The methods employed to concentrate and purify certain of the components will be described later.

Effect of Azo Dyes on Liver Components—The feeding of diets containing *m'*Me-DAB appeared to induce some pronounced changes in certain of the liver components (Table II). The total liver moisture remained relatively constant (75 to 78 per cent) over the 8 week feeding period, as did the total nitrogen. The non-protein nitrogen, however, increased somewhat as a result of feeding the azo dye. An increase was observed in the total phosphorus of the liver which could be accounted for by the increase in the nucleic acid fractions. Of all the components determined, the DNP exhibited the greatest change as a result of administration of the carcinogen. There was a progressive increase, and at 8 weeks this component was almost double that observed in the normal rat liver (266 *versus* 506 mg. per cent). Cirrhosis and other liver damage progressed as the azo dye was fed, and there appeared to be some correlation between increasing liver DNP and cirrhosis. The high liver DNP, after feeding of the carcinogenic diet for 8 weeks, actually approached that observed in hepatomas. It should be noted that the estimation of DNP and also of RNP depends on the colorimetric action of desoxyribose and ribose bound to purine bases after the nucleic acids have been liberated from the protein by hot trichloroacetic acid (method of Schneider (14)). An increase in other components that react with the Dische reagent, for example, would result in apparent high values for DNP. It appears improbable that this was responsible for the high values observed in the livers of rats fed the diet containing azo dye, since an actual increase in the quantity of the DNP fraction could be observed visually when this nucleoprotein complex was precipitated by reducing the NaCl concentration in the 1.0 M extract to approximately 0.14 M. Phosphorus data also support this conclusion. The total of nucleic acids as determined colorimetrically in the initial fraction obtained by extraction with 0.4 M NaCl has been plotted against the protein phosphorus content of this fraction for each of the twenty-two runs (Fig. 1). It may be observed that an increase in nucleic acids is cor-

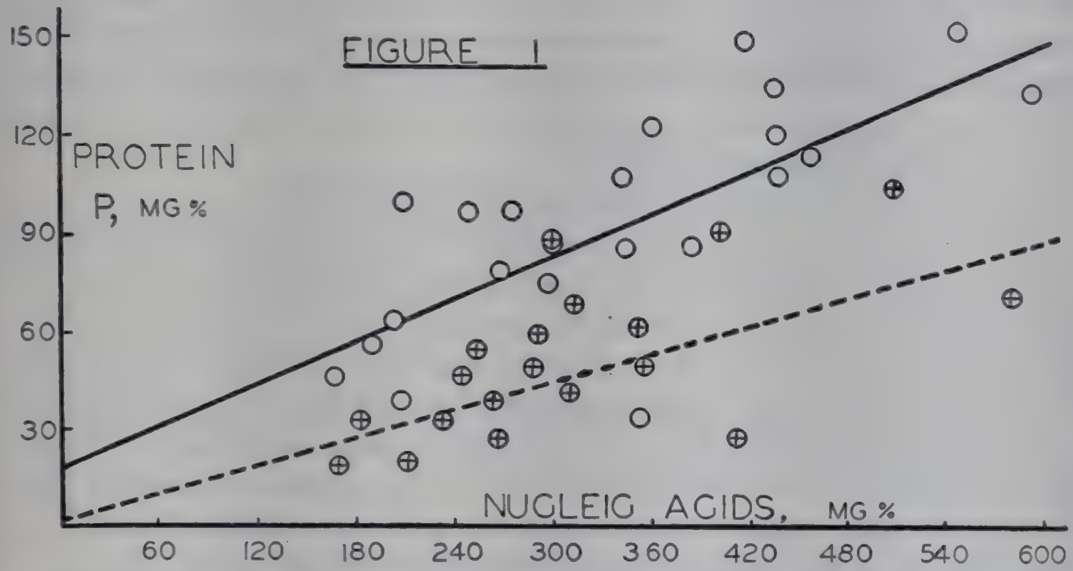


FIG. 1. The protein phosphorus content plotted against the total nucleic acid content of various NaCl extracts of liver, indicating that increases in the amounts of nucleic acids are accompanied by an increased phosphorus content. ○, corresponds to the 0.4 M NaCl extract (mostly ribonucleic acid); ⊗, corresponds to 1.0 M NaCl extracts (mostly desoxyribonucleic acid). The lines are calculated by the method of least squares.

related with an increase in protein phosphorus. Similarly, the phosphorus content of the 1.0 M extract, which contains most of the DNP, was plotted against nucleic acid content. Here again it is evident, from the line of least squares, that increases in nucleic acids are accompanied by an increased phosphorus content. This provided further evidence that the increase in the liver DNP accompanying the feeding of the azo dye is real and not a result of other substances that may react with the Dische reagent.

At present it is difficult to assign any significance to changes in the RNP content (Table II). There was an initial decrease in this component accompanying the feeding of diets containing the azo dye. After feeding the dye for 4 to 6 weeks, however, there was some increase. These observations appear to be in agreement with those of Opie (11), although his studies were on a cytological basis and involved the feeding of the less active carcinogen, *p*-dimethylaminoazobenzene.

The riboflavin content decreased progressively in the liver; after 8 weeks of feeding the diet containing the *m'*Me-DAB, it was approximately 50 per cent of that observed in the livers of rats fed the dye-free basal diet (2466 *versus* 1278 γ per cent). These findings are in agreement with those of other investigators (5, 9). Normal rat liver contains 1.3 per cent globulin and almost the same percentage of albumin. The albumin remained at approximately this level throughout the period of azo dye feeding. An increase was noted, however, in the globulin fraction. This increase appeared within 2 weeks and the globulin content remained at approximately 2 per cent throughout the precancerous period.

In a corresponding study on the effect of azo dye carcinogenesis on serum proteins, it was observed that the serum γ-globulin concentration increased with the feeding of the *m'*Me-DAB.[3] Globulin and albumin were not determined in the liver tumors because of the small amounts of suitable tissue available.

In order to ascertain whether the increase in DNP was specific for the carcinogenic azo dyes or whether this was perhaps a response to azo compounds in general, rats were fed diets that contained the relatively noncarcinogenic compound, azobenzene, at a level of 0.05 per cent (Table III). In this series, the total liver nitrogen and phosphorus contents were somewhat higher than were observed for the basal control diets (Table I). Riboflavin, DNP, and RNP concentrations were essentially normal after the diet containing the azobenzene was fed for either 2 or 4 weeks, indicating that the increase in the DNP content noted in the animals fed the *m'*Me-DAB was associated with carcinogenesis and was not due to azo compounds in general.

[3] Cook, H., Griffin, A. C., and Luck, J. M., *J. Biol. Chem.*, in press.

Purification of Liver Nucleoproteins—Extraction of liver with 0.4 M NaCl resulted in a fraction containing 65 per cent of the total liver RNP and approximately 7 per cent of the DNP. The 1.0 M extract contained 66 per cent of the total DNP and 10 per cent of the RNP (Table I). Almost all of the RNP present in the initial NaCl extract may be sedimented out by adjusting the pH to 5.0 and centrifuging. If this precipitate was stirred in water and the pH adjusted to 8.0, the RNP was dissolved. By repeating this process several times, it was possible to obtain a fraction containing over 50 per cent of the RNP originally present in the liver and from 2 to 5 per cent of the total DNP. This fraction was also free of the more soluble liver components; *i.e.*, albumins, globulins, non-protein nitrogen, and phosphorus. By centrifuging the initial NaCl extract for 4 hours at 100,000 × g in the ultracentrifuge at pH 7.0, an almost complete sedimentation of the RNP was effected. This high speed centrifugation probably sedimented the formed elements of the cytoplasm that contain the RNP (21). Further work is in progress to determine whether ultracentrifugation will aid in the purification of RNP fractions.

TABLE III

Effect of Azobenzene on Liver Components

Purified basal diet + 0.05 per cent azobenzene.

Component	2 wks.	4 wks.
Nitrogen, *mg.* %	2900	2850
Phosphorus, *mg.* %	342	332
Desoxyribonucleoprotein, as desoxyribonucleic acid, *mg.* %	318	273
Ribonucleoprotein, as ribose, *mg.* %	153	170
Riboflavin, γ %	2330	2278
Non-protein nitrogen, *mg.* %	200	199
" phosphorus, *mg.* %	100	102

The values are on a fresh tissue basis, two groups per period, four to six rats per group. Livers appeared normal at the 2 and 4 week periods.

Some preliminary steps were also taken toward further purification of the DNP from the 1.0 M NaCl extract. Mirsky and Pollister (20) precipitated the DNP from this fraction by adjusting the NaCl to 0.14 M, at which concentration the protein appeared in a fibrous state. The fibers were then redissolved in 1.0 M NaCl. This procedure, repeated one or more times, resulted in a preparation of DNP relatively free of the RNP and other components. In the present investigation, purification by precipitation in 0.14 M NaCl resulted in a preparation containing approximately 50 per cent of the DNP and 6 per cent of the RNP originally present in the liver. This fraction also contained 4 to 8 per cent of the total liver nitrogen, approximately 10 per cent of the phosphorus, and had a N:P ratio of 4.0. All the preparations contained some riboflavin, although this constituted only 2 to 3 per cent of the total liver riboflavin in most cases. No studies were made to determine whether a flavoprotein constituted an integral part of the DNP present or whether the riboflavin present was merely residual. A DNP preparation containing relatively little RNP was prepared by precipitation of the fibers in 0.4 M NaCl instead of in physiological saline. Some DNP was lost by precipitation in 0.4 M NaCl because of the increased solubility at this higher salt concentration. The resulting DNP, however, contained less than 2 per cent of the total RNP and had a N:P ratio of 3.8. In this range it becomes difficult to obtain an accurate analysis for the RNP in the presence of large quantities of DNP.

It became evident that a better initial separation of the two nucleoprotein types would facilitate the subsequent purification of these different fractions. It had been observed previously that low temperature storage of liver altered the extractability of certain of the proteins. A quantity of perfused rat liver was divided into three equal portions. One portion was fractionated immediately while the second and third portions were stored at −15° for 2 and 4 weeks respectively. When the tissues stored at the low temperature were extracted with 0.4 M NaCl, more of each component appeared in this fraction. Thus, over 80 per cent of the total nitrogen and phosphorus were extracted, as compared with approximately 55 to 60 per cent of these components in fresh, non-frozen liver (see Table I). Approximately 90 per cent of the RNP was removed after the tissues had been subjected to the low temperature storage as compared to a 65 per cent extraction in non-frozen liver. Perhaps more striking was the removal of riboflavin after the cold treatment. Only 40 per cent or less of the total riboflavin was initially extracted in the non-frozen liver, whereas 90 per cent could be extracted after the liver was frozen for the 2 or 4 week period. Variations as to time of storage, freezing temperature, and methods of freezing were carried out in order to determine whether low temperature would actually improve extractability and the fractionation of the liver proteins. It was found that freezing of the livers for only a few hours at −15° improved the extractability of most components. The same was noted if the liver was frozen in a dry ice-ether (or acetone) mixture for 10 to 15 minutes. Finally a fractionation was carried out wherein the freshly removed liver tissue was immersed in liquid air for 30 minutes. The tissue was then homogenized and twice extracted with 0.4 M NaCl as described under "Methods." The remaining residue was extracted with 6 volumes of 1.0 M NaCl. All centrifugings were made in a Servall supercentrifuge for approximately 20 minutes at 20,000 × *g*. The initial extract with 0.4 M NaCl contained 83 per cent of the total RNP and 4 per cent of the DNP, while the 1.0 M NaCl extract contained 82 per cent of the DNP and 3 per cent or less of the RNP. From these fractions it should be possible to obtain relatively pure preparations of RNP, DNP, albumins, globulins, etc., by the methods previously described.

DISCUSSION

The increase in the liver DNP content appeared to be one of the most significant changes accompanying the administration of the carcinogen, *m*′Me-DAB. Other investigators (9) have also observed that a similar response, though less intense, could be obtained when the azo dye, *p*-dimethylaminoazobenzene, was fed. A relatively non-carcinogenic compound, azobenzene, had seemingly little effect on the liver DNP, suggesting that the above increase was only associated with the process of azo dye carcinogenesis. Hepatic tumors have a considerably higher DNP concentration than normal liver tissue, and in the present investigation it was found that the feeding of the carcinogenic azo dye resulted in a progressive increase in the amount of this component to a level approaching that of actual tumor tissue. Generally, it has been observed that tumor tissues contain more DNP than the normal tissues of tumor origin; however, the concentration of nucleic acids varies considerably in different normal tissues (7). Stowell (22) also has observed a higher cell content of desoxyribonucleic acid in epidermoid carcinoma than in normal human epidermis.

Histological studies were not made on the liver of rats fed the azo dyes. It would appear certain that cellular changes accompany the large increase in DNP in the liver cell nuclei. Schneider (23) observed a cytoplasmic nuclear ratio of 5.85 for normal rat liver and 3.04 for rat hepatomas, indicating that the tumor cells have large nuclei or a greater number of small cells with nuclei of approximately the same size as normal cells. Our own observations reveal only an over-all increase in the total liver DNP and provide no information as to whether it is a result of enlarged cell nuclei, of an increased number of small cells, or of an increase in DNP content per unit volume with no change in nuclear or cell size. There was a decrease in the amount of liver riboflavin with the feeding of azo dyes which is also in agreement with the findings of other investigators (3–5). This decrease in liver riboflavin appears to be roughly proportional to the increase that occurs in the DNP. Both components in the precancerous livers approach the levels found in hepatomas, which may be of significance in the actual process of carcinogenesis. It is difficult to compare normal or even precancerous liver with liver tumors without a consideration of the type and uniformity of tissues involved. In the present study, more emphasis was placed on the precancerous liver changes, although it appears significant that certain components of the precancerous livers do approach the levels found in liver tumors.

SUMMARY

1. The livers from normal rats and from those fed diets containing the carcinogenic azo dye, *m'*-methyl-*p*-dimethylaminoazobenzene, were fractionated into several protein components on a basis of differential solubility in NaCl solutions. Quantitative analyses were made for nitrogen, phosphorus, desoxyribonucleoproteins, ribonucleoproteins, riboflavin, albumin, globulin, and non-protein nitrogen and phosphorus in the appropriate fractions.

2. Methods of purifying the ribo- and desoxyribonucleoproteins are described. Freezing of liver tissue before fractionation improved the extractability of most of the liver proteins.

3. The amount of desoxyribonucleoproteins increased progressively and approached the hepatoma level as the diet containing this azo dye was fed. The normal level was 266 mg. per cent (as desoxyribonucleic acid); after receiving azo dye for 8 weeks it was 506 mg. per cent; the level in liver tumors was 756 mg. per cent. The liver riboflavin content decreased as the azo dye was fed; the globulin content increased significantly, while concentrations of other components remained relatively normal.

4. A relatively non-carcinogenic azo compound, azobenzene, had no appreciable effect on any of the components determined.

We wish to acknowledge with thanks the receipt of grants-in-aid from the American Cancer Society and the United States Public Health Service. The assistance of Eleanore Frey and Carol Moore in conducting many of the analyses incident to these studies is also gratefully acknowledged. The histological studies referred to were very kindly made by Dr. H. Kirkman of this University.

BIBLIOGRAPHY

1. Hoch-Ligeti, C., *Cancer Res.*, **7**, 148 (1947).
2. Schneider, W. C., and Potter, V. R., *Cancer Res.*, **3**, 353 (1943).
3. Kensler, C. J., Sugiura, K., and Rhoads, C. P., *Science*, **91**, 623 (1940).
4. Miller, J. A., *Ann. New York Acad. Sc.*, **49**, 19 (1947).
5. Griffin, A. C., and Baumann, C. A., *Arch. Biochem.*, **11**, 467 (1946).
6. Miller, E. C., and Miller, J. A., *Cancer Res.*, **7**, 468 (1947).
7. Schneider, W. C., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **12**, 169 (1947).
8. Greenstein, J. P., Biochemistry of cancer, chap. 7 (1947).
9. Price, J. M., Miller, E. C., and Miller, J. A., *J. Biol. Chem.*, **173**, 345 (1948).
10. Masayama, T., and Yokoyama, T., *Gann*, **34**, 174 (1940).
11. Opie, E. L., *J. Exp. Med.*, **84**, 91 (1946).
12. Miller, J. A., and Baumann, C. A., *Cancer Res.*, **5**, 227 (1945).
13. Rusch, H. P., Baumann, C. A., Miller, J. A., and Kline, B. E., in Moulton, F. R., American Association for the Advancement of Science research conference on cancer, Washington, 267 (1945).

14. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).
15. Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, **37**, 354 (1943).
16. King, E. J., *Biochem. J.*, **26**, 292 (1932).
17. Conner, R. T., and Straub, G. J., *Ind. and Eng. Chem., Anal. Ed.*, **13**, 385 (1941).
18. Andrews, J. S., *Cereal Chem.*, **20**, 3 (1943).
19. Robinson, H. W., and Hogden, C. G., *J. Biol. Chem.*, **135**, 707 (1940).
20. Mirsky, A. E., and Pollister, A. W., *J. Gen. Physiol.*, **30**, 117 (1946).
21. Claude, A., *J. Exp. Med.*, **84**, 61 (1946).
22. Stowell, R. E., and Cooper, Z. K., *Cancer Res.*, **5**, 295 (1945).
23. Schneider, W. C., *Cancer Res.*, **5**, 717 (1945).

SERUM VASOCONSTRICTOR (SEROTONIN)

III. CHEMICAL INACTIVATION*

By MAURICE M. RAPPORT,† ARDA ALDEN GREEN, and IRVINE H. PAGE

(*From the Research Division of the Cleveland Clinic Foundation, Cleveland*)

(Received for publication, July 17, 1948)

The preparation of active concentrates of serum vasoconstrictor in a stable form (1) made possible the study of the active principle by means of inactivation measurements. This work was begun with three purposes in mind: first, to acquire information on the sensitivity of the active principle to various reagents in order to reduce losses occurring during isolation; second, to learn some of its chemical characteristics before attempting further purification; third, and most important, to determine which chemical reagents are most suitable for abolishing the vasoconstrictor activity of serum.

EXPERIMENTAL

Effect of Reagents—Since the extent of inactivation is dependent on the rate of reaction, and therefore on temperature and concentration of reactants as well as time, these factors were controlled. Samples of purified serum vasoconstrictor were heated for 5 minute periods at 97° with equal volumes of the reagent at various concentrations, until one was found which gave 25 to 75 per cent inactivation. At this concentration, samples were heated for varying periods of time (*e.g.*, 3, 6, 9, 12 minutes) at 97° until no increase in inactivation occurred with continued heating. Under these conditions of time and concentrations of reactants, the constrictor substance was treated with varying amounts of the reagent, and the extent of inactivation was determined.

Experiments were set up as follows. To 0.2 ml. of a solution of purified serum vasoconstrictor containing 1 mg. per ml. (750 units per ml.)[1] was added 0.05 to 0.30 ml. of the reagent solution. These solutions were heated in a boiling water bath (95–97°) for a fixed time, cooled, and made up to 3.0 ml. with distilled water. Their constrictor activity in the rabbit

---

* This study was supported in part by a grant from the United States Public Health Service, Cardiovascular Study Section.

† Present address, Department of Medicine, Columbia University, College of Physicians and Surgeons, New York.

[1] The unit has been defined (1) as the response given in the rabbit ear preparation by 0.2 ml. of a solution containing 1.2 γ per ml. of a purified serum vasoconstrictor preparation.

ear preparation was then compared as described (1) with controls containing no reagent. No loss of activity was observed in the controls. Reagent controls were performed; it was found that, at the concentrations employed, most of them did not exert significant effects.

With several reagents examined according to this scheme, the extent of inactivation was directly proportional to the quantity of reagent employed, while with others it was not (Table I). With still others, only qualitative observations were made, either because the reagents could not be adapted to the method described or because they did not appear to be sufficiently effective (Table II).

TABLE I

Inactivation Reagents Studied Quantitatively

97°, 5 minutes, pH 4 to 6.

Reagent	Concentration	Quantity to inactivate 100 units of purified vasoconstrictor
	moles per l.	*micromole*
Potassium persulfate	4×10^{-5}	0.006
Iodine*	5×10^{-5}	0.006
Potassium permanganate†	5×10^{-6}	0.005
Sodium bisulfite	1×10^{-4}	0.02
Pyridine perbromide hydrobromide	5×10^{-4}	0.04
Potassium ferricyanide	5×10^{-4}	0.06
Chloramine-T	5×10^{-4}	0.15
Ceric sulfate‡	1×10^{-4}	~0.01
Potassium periodate‡	1×10^{-4}	~0.02
Iodic acid‡	5×10^{-4}	~0.05

* 3 minutes; 25°, 60 minutes.

† 25°, 10 minutes.

‡ Extent of inactivation not proportional to quantity of reagent.

The three most effective inactivating reagents have been tried on pure serotonin. Under closely similar conditions, within an experimental error of 20 per cent, the same quantity of iodine (or potassium persulfate) was required to inactivate 100 units of either the concentrate or pure serotonin. With potassium permanganate, twice as much reagent was required with the less pure material.

Effect of pH—The inactivating effect of alkali was found to be much more rapid than that of acid (Table III). The results obtained with acid were variable until the experiments were performed under nitrogen.

Because of the sensitivity of the serum vasoconstrictor to alkali, strongly alkaline reagents were not tried. Moreover, since the purified preparation was soluble only in water, non-aqueous reagents were not employed. The pH values of all inactivation reactions were below 7, most of them being between pH 4.5 and 6.0.

Reagents found to be ineffective as inactivating agents are listed in Table IV.

TABLE II

Inactivation Reagents Studied Qualitatively

Reagent	Conditions for inactivation of >50%
Potassium dichromate	0.005 M, 37°, 3 hrs.
Ferric chloride	0.001 " 97°, 10 min.
Hydrogen peroxide	30% solution, diluted 1:1000, 97°, 6 min.
Hydroxylamine hydrochloride	0.001 M, 97°, 15 min.
Sodium nitrite*	0.005 " 25°, 3 hrs.
Nitranilic acid	0.01 " 97°, 10 min.
Auric chloride†	4%, 25°
Chloroplatinic acid	10%, 25°
Mercuric sulfate‡	10% in H_2SO_4, 25°
Norit§	25°, 5 min.
Amberlite IR-100-H§	25°, 5 "

* Reagent caused vasodilatation.

† Inactivation performed on pure serotonin.

‡ The activity was precipitated. Recovery after removal of Hg^{++} with H_2S has not exceeded 20 per cent.

§ These adsorbents are listed because both are effective in removing activity, none of which has ever been eluted under a wide variety of conditions.

TABLE III

*Inactivating Effect of pH on Purified Serum Vasoconstrictor at 97°**

pH	Time	Per cent inactivation
	min.	
4.5-6.5	360	0
1.5	360	0
0.5	240	30-40
7.5	30	20
10.3†	10	>90

* Under nitrogen.

† No inactivation in preceding 30 minutes at 25°.

DISCUSSION

Inactivation studies are difficult to appraise as evidence for the existence of chemical groupings. It is clear, as has been pointed out (2), that a positive result indicates change in the structure of the molecule, while lack of inactivation does not necessarily preclude such change. When impure preparations are employed, an additional difficulty arises; namely, inactivation may be due to a product of the reaction between the reagent and an impurity. Even more ambiguous is the reasoning that certain reagents react *only* with specific chemical groupings. Carrying out the inactivation studies on a quantitative basis, and in such a way that minimal handling was required before bioassay, made it easier to evaluate the findings.

It is believed, on the basis of the results so far obtained, that serotonin has a sufficiently sensitive structure to permit most "classification" reagents to cause inactivation under relatively mild conditions. However, because of this sensitivity to such a variety of reagents at relatively high dilutions, it does not seem justifiable as a result of these studies alone to draw conclusions as to the presence of specific chemical groupings.

TABLE IV

Reagents Causing No Inactivation under Conditions Employed

Reagent	Conditions
Ferrous sulfate	0.005 M, 97°, 10 min.
Silver nitrate	0.005 " 97°, 10 "
Ninhydrin*	0.005 " 97°, 12 "
Formaldehyde	40% solution, diluted 1:1000, 97°, 12 min.
Cupric sulfate	0.001 M, 97°, 10 min.
Cuprous oxide†	25°
p-Chloromercuribenzoic acid‡	25°
Diazomethane§	25°
2,4-Dinitrophenylhydrazine	Suspension, 37°, 90 min.
Amberlite IR-4B	25°, 60 min.

* Under these conditions, 30 per cent inactivation of a sample of pure serotonin was observed. The reagent has a weak constrictor effect.

† Precipitate formed. The supernatant retained full activity.

‡ Kindly supplied by Dr. T. P. Singer, Western Reserve University.

§ Performed in methanolic solution on pure serotonin. Nitrogen liberated; no activity lost.

Conditions may ultimately be found under which the reagents in Table I will cause relatively specific inactivation of serotonin. This requires determination of the mildest conditions which are still effective. For example, it has been found that iodine is as effective at room temperature as at 97°, although the minimal time requirement, which is less than 1 hour, must still be determined.

The fact that iodine completely destroys the activity, and that the same quantity of iodine destroys the same number of units of activity of both pure serotonin and the concentrate only 0.75 per cent pure,[2] indicates that the total activity of the impure preparation is due, probably, to a single substance, namely serotonin. Additional evidence comes from the correlation of activity and color produced in the Ehrlich reaction[3] in the course of further purification. If the sensitivity of the serotonin molecule is considered in conjunction with its relatively high (25 to 35 per cent) recovery from serum in the concentrate, the conclusion seems justified that the vasoconstrictor property of serum, at least as measured in the rabbit ear preparation, is almost entirely due to serotonin.

The authors wish to acknowledge the valuable assistance of Mr. John M. Means and Miss Elizabeth Hunt in this investigation.

SUMMARY

In order to determine which reagents were most suitable for abolishing the vasoconstrictor activity of serum, the inactivation of a beef serum vasoconstrictor (serotonin) concentrate by chemical reagents was studied. Certain oxidizing and halogenating agents, especially potassium persulfate, potassium permanganate, and iodine, were found to be effective at high dilution. By comparing the quantity of iodine required for inactivation of the concentrate and pure serotonin, evidence was obtained favoring the view that serotonin is solely responsible for the vasoconstrictor activity of serum.

BIBLIOGRAPHY

1. Rapport, M. M., Green, A. A., and Page, I. H., *J. Biol. Chem.*, **174,** 735 (1948).
2. Brown, G. B., and du Vigneaud, V., *J. Biol. Chem.*, **141,** 85 (1941).

---

[2] The activity of pure serotonin is 100,000 units per mg. The concentrate contains 750 units per mg.

[3] Unpublished data (see Paper IV for the method).

SERUM VASOCONSTRICTOR (SEROTONIN)

IV. ISOLATION AND CHARACTERIZATION*

By MAURICE M. RAPPORT,† ARDA ALDEN GREEN, AND IRVINE H. PAGE

(From the Research Division of the Cleveland Clinic Foundation, Cleveland)

(Received for publication, July 17, 1948)

In the first paper of this series (1) the partial purification of a vasoconstrictor substance from beef serum was reported. Further purification of the active substance led to its isolation. A preliminary report of the method together with analytical and pharmacological data has also been published (2). It is the purpose of this paper to present the details of the method of isolation, the more complete analyses now at hand, and some further characterizations of the substance.

The problem of isolating this principle presented the difficulties usually encountered in vitamin research (3) in that the substance was present in the source material (beef serum) in a dry weight concentration of about 0.005 per cent. Initial obstacles were overcome with the preparation of a stable concentrate which was 0.8 per cent pure and contained 25 to 35 per cent of the activity originally present in the serum (the percentages are calculated from the activity of the pure substance).

This concentrate was composed mainly of the ammonium salt of 5-nitrobarbituric acid (the precipitating agent). The active principle appeared to be involved in this concentrate in some kind of complex, since repeated recrystallization did not effect important changes in activity of the crystals so obtained. After many procedures which resulted in large losses of activity were tried, it was found possible to separate 90 per cent of the inactive materials from the concentrate with a loss of less than 5 per cent of the total activity by precipitating them with acetone from hot aqueous solution. Concentration of the filtrate and recrystallization of the residue from methyl alcohol led to the isolation of a crystalline substance with vasoconstrictor action in the rabbit ear preparation approximately twice that of commercial epinephrine.

EXPERIMENTAL

Melting points were determined on the Kofler micro hot stage and are corrected.

---

* This study was supported in part by a grant from the United States Public Health Service, Cardiovascular Study Section.

† Present address, Department of Medicine, Columbia University, College of Physicians and Surgeons, New York.

Isolation—28 gm. of concentrate, prepared as described (1) from about 210 liters of beef serum (450 liters of blood) and containing 750 units[1] of activity per mg., were dissolved in 3.4 liters of boiling distilled water. 3.5 liters of reagent grade acetone were then added to the hot (80°) solution, resulting in the immediate formation of a copious, finely divided precipitate.

For convenience of operation, especially with regard to minimizing the time of heating and reducing the hazards attending the addition of the acetone to a solution whose temperature was above the boiling point of acetone, this procedure was carried out in separate portions by employing one-seventh the quantities listed above, which were then combined.

After chilling the mixture in the cold room overnight, the colorless precipitate was filtered by suction and washed with 50 per cent aqueous acetone. This precipitate weighed 24.0 gm. and assayed at 25 units per mg. The filtrate was then evaporated below 50° at reduced pressure under nitrogen to a volume of 245 ml. The precipitated material resulting from this evaporation was dissolved by boiling the solution, the addition of 55 ml. of water being required. 350 ml. of acetone were then added, the mixture was left in the cold room 2 days, and then suction-filtered, giving 1.64 gm. of buff-colored precipitate assaying at 200 units per mg. The filtrate (containing 18 million units) was then evaporated to dryness as before. The residue was extracted with 50 ml. of 50 per cent aqueous methanol. This extract was evaporated to dryness, and the residue was extracted with 10 ml. of hot absolute methanol. On cooling, this extract deposited brownish yellow rosettes of prisms. The supernatant was decanted, and the crystals were washed with methanol and acetone by decantation. After drying, the crystals weighed 143 mg., m.p. 196–201° (decomposition); assay 80,000 units per mg.

The crystals were recrystallized by dissolving them in 10 ml. of water and, at 60°, adding 35 ml. of acetone, giving, after washing and drying, 108 mg. of light buff-colored, thin rhomboid plates, m.p. 207–212° (decomposition); assay 100,000 units per mg. By adding 50 ml. of acetone, a second crop of 12.5 mg., m.p. 201–205° (decomposition), was obtained from the filtrate.

The first crop material was again recrystallized from 6 ml. of water and 18 ml. of acetone to give 93 mg. of light buff platelets, m.p. 209–212° (decomposition); assay 100,000 units per mg. By adding 75 ml. of acetone to the mother liquor, a second crop of 9.5 mg., m.p. 204–208° (decomposition), was obtained.

---

[1] The unit of activity has been defined (1) as the response given in the rabbit ear preparation by 0.2 ml. of a solution containing 1.2 γ per ml. of a purified serum vasoconstrictor preparation.

The result described above represents the last "large scale" isolation effort and is the best one. The procedure has been carried out twice on the scale described, and several other times with about one-tenth this quantity of concentrate. In each experiment, the pure active substance has shown the same physical and chemical properties. One of the two complications encountered in the less straightforward experiments was the presence of an impurity which had the same solubility characteristics in water, methanol, and acetone as the active substance and appeared to be inorganic, with a melting point near 290°. The other was the formation of diliturate complexes of variable composition (as revealed by ultraviolet absorption spectra) which were deposited by the methanol extract and could not be substantially purified by several recrystallizations from methanol.

Variations from the procedure described which were employed in the previous experiments may have contributed to their less satisfactory results. Lack of additional concentrate prevented clarification of this point.

Melting Point—This is a decomposition point, slight effervescence occurring at and slightly above it. The discoloration of the sample is slight and the melt is clear. The sharpest decomposition point, obtained on the first analytical sample, was 212–214°. The same sample, after 3 weeks, melted at 206–209°. The melting points were taken by a uniform procedure. Heating was begun below 50°, carried to within about 20° of the melting point at 6–8° per minute, and at the decomposition point the rate of heating was 2–2.5° per minute.

Elementary Analyses—Two samples, prepared from different batches of concentrate, were analyzed by two analytical laboratories.

$C_{14}H_{23}O_7N_5S$ (405.4).	Calculated.	C 41.47, H 5.72, N 17.28, S 7.91, N-CH_3 3.71
Sample I, m.p. 212–214°.[2]	Found.	" 41.38, " 6.03, " 16.97
" II, " 209–212°.[3]	"	" 41.75, " 5.87, " 17.63, S 8.03, N-CH_3 2.99

An analysis for ionic sulfate, performed according to the nephelometric procedure of Hoffman (4) on 0.50 and 0.75 mg. of Sample II, gave 21 and 23 per cent sulfate. $C_{14}H_{21}O_3N_5 \cdot H_2SO_4$ requires 23.7 per cent. The isolated material thus appears to be the sulfuric acid salt of an organic base.[4]

Solubility—Serotonin is soluble in water to the extent of 100 mg. per ml. at 50° and about 20 mg. per ml. at 27°. The substance is also soluble in glacial acetic acid. The pure material is very sparingly soluble in methanol and 95 per cent ethanol, and insoluble in absolute ethanol, acetone, pyridine, chloroform, ethyl acetate, ether, and benzene.

---

[2] Analyses by E. Thommen, Basel.

[3] Analyses by A. Elek, Los Angeles.

[4] The name "serotonin" which was proposed for the isolated substance (2) should properly be reserved for the free base. The isolated material would then be serotonin sulfate. Since only the sulfate is considered in this communication, serotonin, when used, refers to the sulfate.

Optical Activity—A solution of 10.4 mg. of serotonin in 1.98 ml. of water showed no significant rotation at 31° in a 2 dm. tube.

Ultraviolet Absorption Spectrum—The absorption spectrum of serotonin in aqueous solution at pH 3.5 has a maximum at 2750 A, a shoulder with a point of inflection at 2930 A, and a minimum at 2500 A (Fig. 1). For purposes of comparison, the absorption spectrum of tryptophan (Eastman Kodak) in water at pH 3.6 is also presented. With respect to the location of maxima and points of inflection, neither of the curves shows significant change at pH 10.3.

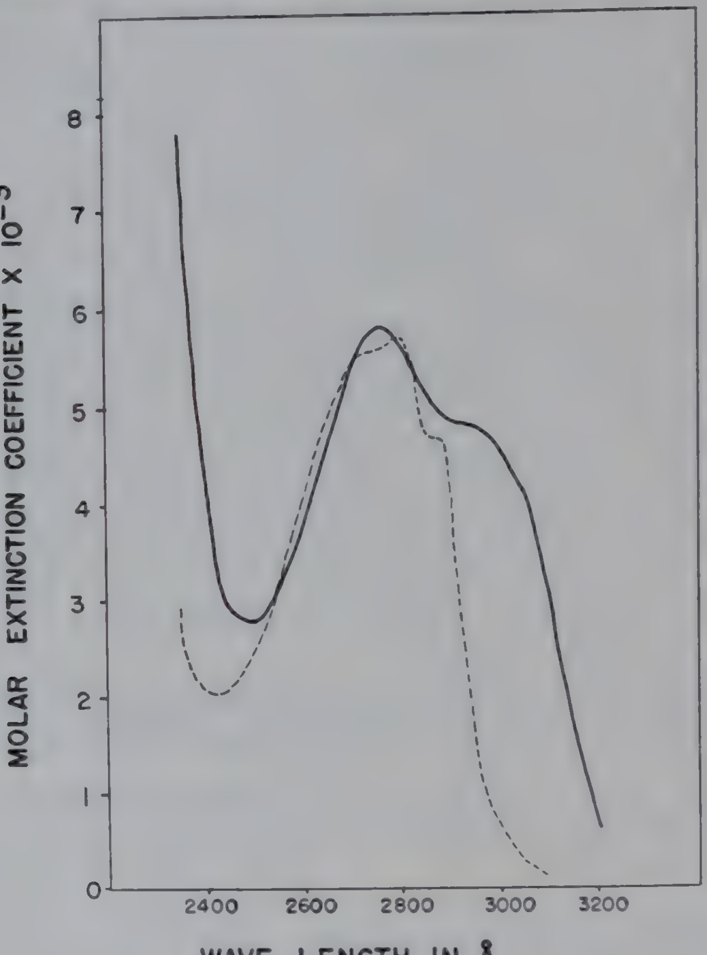


FIG. 1. Ultraviolet absorption spectra of serotonin (solid line) and tryptophan (dotted line) in water at pH 3.5.

Potentiometric Titration—21.5 mg. of serotonin were dissolved in 5.0 ml. of water and titrated with 0.0157 N carbonate-free sodium hydroxide at 26°. pH values were determined to the nearest 0.05 unit with a Beckman pH meter (glass electrode). Moles of acid or base combined per mole of serotonin were calculated, assuming a molecular weight of 405 for serotonin. The experimental points are plotted in Fig. 2. The line is the theoretical dissociation curve for pK′$_1$ of 4.9 and pK′$_2$ of 9.8. The titration was not carried beyond pH 9.7 because of the inaccuracy of the electrode in this range and in order to prevent possible inactivation of the serotonin.

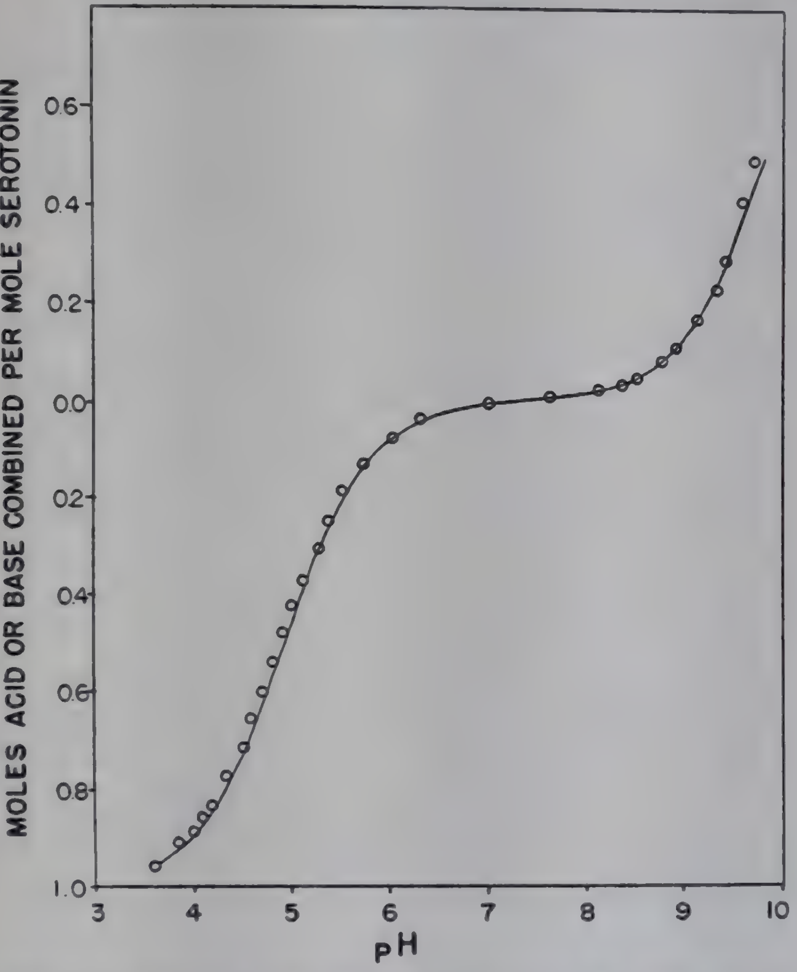


Fig. 2. Dissociation curve of serotonin. The points are experimental, the curve theoretical, based on the constants pK′$_1$ = 4.9 and pK′$_2$ = 9.8.

Under identical conditions, 13.3 mg. of tryptophan were titrated. From the curve obtained, pK′$_1$ of 2.4 and pK′$_2$ of 9.4 were estimated, in good agreement with the published values of 2.38 and 9.39 (5).

Color Reactions and Classification Tests—Serotonin gives positive Hopkins-Cole, Ehrlich, Folin, and pine splinter tests.

In the Shaw-MacFarlane modification (6) of the Hopkins-Cole test with glyoxylic acid, 1 mole of serotonin gives a color equivalent to 0.89 mole of tryptophan. Visually, the color is violet-blue instead of the violet obtained with tryptophan. Without the glyoxylic acid, a pink color is developed by serotonin under these conditions.

In a modification[5] of the procedure of Graham *et al.* (7) for the Ehrlich *p*-dimethylaminobenzaldehyde reaction, 1 mole of serotonin gives a color equivalent to 1.10 moles of tryptophan. The final color is very similar for the two substances.

In the Folin-Ciocalteu reaction (8), 1 mole of serotonin gives a color equivalent to 2.4 moles of tryptophan.

The Sakaguchi reaction (9), the Bratton-Marshall method for diazotizable amines (10), the Gerngross-Voss-Herfeld reaction (11), and the Pauly diazo reaction for imidazoles (12) were all negative. In the latter two reactions, yellow colors were obtained containing no trace of red.

Qualitative ninhydrin, Molisch, and ferric chloride tests were negative. Serotonin heated with ninhydrin-sodium acetate produced a chrome yellow color.

The pine splinter test served the purpose of a rapid semiquantitative assay in the final stages of the isolation when carried out in the following way. 2 drops of concentrated hydrochloric acid were placed on a tongue depressor and spread with a glass slide. When the excess liquid had soaked into the wood, a trace of the sample to be tested was rubbed on the prepared wood surface. Fractions having an activity of 35,000 units or more per mg. gave a deep red color. The activity of fractions which still gave a detectable test was about 10,000 units per mg. (10 per cent pure).

Serotonin reduces ammoniacal silver nitrate, but the reaction is not a typical aldehyde response. In a test performed on 50 γ of substance, a brown color formed within 1 minute, giving way to a red-brown precipitate in several hours, in turn followed by a black precipitate with slight mirror formation in 24 hours, the supernatant solution remaining reddish in color. With silver nitrate at pH 5, a slight reddish color and a black precipitate were observed after about 12 hours. The reaction was probably the same in both cases, occurring more rapidly under basic conditions.

With 10 per cent mercuric sulfate in 2.5 N sulfuric acid, serotonin, at a concentration of 1 mg. per ml., immediately forms a yellow precipitate which is very insoluble in water.

Despite the high percentage of nitrogen, it has not been possible to obtain a stable crystalline picrate with serotonin.

Solid serotonin catalyzes the decomposition of the iodine-azide complex in the Feigl test (13). In solution, however, at a concentration of 1 mg.

---

[5] To 0.5 ml. of the test solution, 1 drop of 2.5 per cent dimethylaminobenzaldehyde in 10 per cent H_2SO_4, 1 drop of 2 per cent $NaNO_3$, and 2.8 ml. of concentrated HCl are added. After standing at room temperature for 20 minutes, 7.0 ml. of 50 per cent (by volume) ethanol are added. The color is read with a No. 54 filter in the Klett-Summerson colorimeter.

per ml., the test is negative. A positive result is reportedly attributable to thio ketones and mercaptans; but since all the sulfur in serotonin appears to be present in the form of sulfate, an impurity may be responsible for the observed result.

Serotonin rapidly reacts with iodine in aqueous solution to give a water-insoluble compound. It does not give the typical tryptophan reaction with bromine in aqueous solution, although intensification of color has been observed in methanol solution.

DISCUSSION

The absorption spectrum and color reactions of serotonin are strongly indicative of the presence of an indole nucleus. This evidence, in combination with biogenetic considerations, suggests a structure which may be closely related to tryptophan or tryptamine. However, pronounced differences are apparent, and the calculated empirical formula focuses attention on two especially interesting aspects of structure; namely, the high nitrogen content and the high degree of saturation.

It is reasonable to assume that both of the groups revealed in the dissociation curve are basic in nature, since a sulfate salt is under consideration whose 0.01 M solution is at pH 3.6. Further, the absence of marked acidic properties, as revealed by lack of inactivation by diazomethane and non-adsorption on Amberlite IR-4B (14), supports this assumption. If, then, the two groups are basic, the stronger ($pK'_b = 4.2$) approximates the base strength of trimethylamine and is what might be expected for a β-aryl ethylamine such as tryptamine, while the weaker ($pK'_b = 9.1$) has the basicity of aromatic amines such as aniline ($pK'_b = 9.4$) or N,N'-disubstituted guanidines such as creatinine ($pK'_b = 9.2$). A third nitrogen can be accounted for in the indole nucleus. To speculate on the disposition of the remaining two nitrogens, they may be associated with the more weakly basic grouping in a disubstituted guanidine structure, or one or both may be combined in some grouping even less basic such as an amide. The formation of complexes with the amide type structure of dilituric acid and the ease of formation of ammonia on treatment with aqueous alkali[6] are consistent with these alternatives.

No primary amino groups are believed to be present in the molecule. The negative diazotizable amine test rules out *aromatic* primary amino groups, while the failure to form any red or violet coloration with ninhydrin-sodium acetate would seem to do the same for *aliphatic* primary amines.

The absence of quaternary nitrogen is indicated by the distillation, in a microsublimation apparatus, of a small sample of the free base, with apparent retention of full activity.[7]

---

[6] Unpublished experiments.

Nothing is yet known of the disposition of the oxygen functions.

From the standpoint of chemical and pharmacological activity, the marked reducing power of serotonin, as evidenced by the reduction of gold, silver, and platinum salts, seems worth emphasizing. The results obtained by inactivation studies (14) would appear to be explained by this property in conjunction with the presence of the indole fragment. The reduction of the Folin reagent by serotonin to the extent of 2.4 times the reduction by tryptophan, mole for mole, indicates the presence of a second reducing group in addition to the indole nucleus. Epinephrine, the second most active constrictor substance in the perfused rabbit ear vessels, causes a mole for mole reduction of 3.0 times the reduction by tryptophan.

The similarity in chemical and biological activity of serotonin and epinephrine is apparent. The further *structural* similarity (one contains the indole ring system, the other is closely related to it) suggests the possibility that clarification of the structure of serotonin may reveal a more general relation between chemical structure and vasoconstrictor action than has been obtained by studying modifications of epinephrine structure.

We wish to acknowledge the valuable assistance of Miss Elizabeth Hunt and Mr. John Means, and to thank Dr. Hans Hirschmann of Western Reserve University for cooperation in determining the optical activity.

SUMMARY

Details of the method of isolating crystalline beef serum vasoconstrictor (serotonin) from a purified concentrate are presented. The color reactions and ultraviolet absorption spectrum indicate the presence of an indole nucleus in the structure. The structural implications of evidence based on color reactions and potentiometric titration are discussed.

BIBLIOGRAPHY

1. Rapport, M. M., Green, A. A., and Page, I. H., *J. Biol. Chem.*, **174**, 735 (1948).
2. Rapport, M. M., Green, A. A., and Page, I. H., *Science*, **108**, 329 (1948).
3. Folkers, K., in Green, D. E., Currents in biochemical research, New York (1946).
4. Hoffman, W. S., Photelometric clinical chemistry, New York, 244 (1941).
5. Schmidt, C. L. A., Appleman, W. K., and Kirk, P. L., *J. Biol. Chem.*, **85**, 137 (1929–30).

---

[7] Unpublished experiment. The oily distillate obtained (at 140°, 0.15 mm.) was dissolved in water. The retention of activity was judged by the correlation of the activity and the ultraviolet absorption spectrum of the solution.

6. Block, R. J., and Bolling, D., The amino acid composition of proteins and foods, Springfield, 91 (1945).
7. Graham, C. E., Smith, E. P., Hier, S. W., and Klein, D., *J. Biol. Chem.*, **168**, 711 (1947).
8. Hawk, P. B., and Bergeim, O., Practical physiological chemistry, 11th edition, Philadelphia, 755 (1937).
9. Jorpes, E., *Biochem. J.*, **26**, 1505 (1932).
10. Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., and Graber, M., *J. Clin. Invest.*, **24**, 388 (1945).
11. Thomas, L. E., *Arch. Biochem.*, **5**, 175 (1944).
12. Jorpes, E., *Biochem. J.*, **26**, 1507 (1932).
13. Feigl, F., Qualitative analysis by spot tests, 3rd edition, New York, 353 (1946).
14. Rapport, M. M., Green, A. A., and Page, I. H., *J. Biol. Chem.*, **176**, 1237 (1948).

SEPARATION AND DETERMINATION OF THE AMYLOSE AND AMYLOPECTIN FRACTIONS OF STARCH

By K. G. KRISHNASWAMY and A. SREENIVASAN

(*From the Department of Chemical Technology, Bombay University, Bombay, India*)

(Received for publication, July 14, 1948)

Most of the earlier procedures used for separating starch into amylose, the linear unbranched component, and amylopectin, the branched chain component (1, 2), have involved degradation and hydrolysis of the starch molecules. During the last few years, however, a number of methods have been proposed which fulfil, more or less, the necessary requirement of protecting the starch constituents from degradation. The more important of these methods for the separation of amylose from starch is based on its selective diffusibility in water at 60° or 80° (1, 3), precipitability with butanol (4), thymol (5), or nitroparaffins (6), and adsorbability on cellulose (7). It is shown in this communication that these methods fail to effect clear-cut as well as quantitative separation of the two starch fractions, while the purity of the products obtained is also variable. It has been possible, by suitable combination of certain of these procedures, to prepare amylose and amylopectin, judged for their purity by the intensity of their iodine colorations under standard conditions, and to determine their exact percentages in any starch preparation by reference to a calibrated curve for intensity of iodine coloration with known mixtures of the pure fractions (3).

EXPERIMENTAL

Preparation of Starch—The major part of the studies reported here was carried out with a sample of starch prepared from a local variety of peas (*Pisum sativum*). The seeds, softened by soaking overnight in water, were ground to a not too fine consistency and the mash was extruded through a cloth bag into a sufficient volume of distilled water. The residual pulp was mashed and pressed out a second time. The combined extract was let stand and the sludge which separated was purified of proteinaceous material by repeated agitation and settling. The starch suspension was finally kneaded through muslin into water and centrifuged to separate the starch, which was washed successively with 20 and 80 per cent ethanol and allowed to dry at room temperature (28°). Analysis of the product gave 0.88 per cent protein, 0.25 per cent ether extractive, and 12.75 per cent moisture.

Determination of Iodine Coloration—The intensity of color developed in a 2 mg. per cent solution of starch or of the various starch fractions, on addition of a solution of iodine in potassium iodide to a final concentration of 4 mg. per cent of iodine, was measured in a 10 mm. cell by a Klett-Summerson photoelectric colorimeter with Filter K_{66} in position (3). The colorimeter was initially adjusted so that the blank, which had a light yellow color due to the iodine in solution, gave a zero reading; the color measurements recorded are in terms of scale readings in the instrument.

Fractionation of Starch by Selective Extraction of Amylose with Hot Water—5 gm. of air-dry starch, mixed with water to avoid lump formation, were treated with about 300 ml. of water at 60° and the suspension maintained at this temperature for 4 hours with slow stirring. It was then centrifuged at 3000 R.P.M. and the supernatant passed through a sintered glass No. 4 filter, which was found more convenient to use than filter paper coated with Hyflo Super-Cel, as recommended in the original procedure (3). The clear filtrate, after addition of methanol to a concentration of 20 per cent volume per volume, was let stand for 48 hours. At the end of this period, the precipitated amylose (Fraction I) was filtered through a sintered glass No. 4 crucible, washed with 95 per cent ethanol, and finally with absolute alcohol before drying in a vacuum oven. The filtrate was further treated with methanol to 50 per cent volume per volume strength and allowed to settle as before. The precipitate (Fraction II) was filtered, washed, and dried to constant weight.

TABLE I

Extraction of Starch with Water at 60°

	On dry basis			Intensity of iodine color (scale readings)*		
	Yield 1	Yield 2	Yield 3	Yield 1	Yield 2	Yield 3
	per cent	*per cent*	*per cent*			
Original starch				143	149	148
Fraction I	14.4	14.7	15.8	279	271	269
" II	0.9	0.6	Trace	181	180	
" III	84.5	85.3	83.5	101	103	100

* Klett-Summerson colorimeter.

The gelatinous residue remaining after the centrifuging of the aqueous starch suspension was ground well, dehydrated by repeated additions of alcohol, filtered, and dried *in vacuo* (Fraction III). Table I gives a set of typical results obtained together with the iodine colorations of the starch and of the different fractions.

In the set of experiments given in Table II, the temperature of fractionation was kept at 80°, as recommended by Meyer (1); the procedure was otherwise the same as that described above.

Fractionations of pea starch and of the crude amylopectin (Fraction III, Table I) were also attempted by treatment for 48 hours at room temperature with 1:2 chloral hydrate solution in water, as recommended by Meyer. The products obtained gave iodine coloration averaging 74 and 67 respectively; by using chloral hydrate solution at 80°, the corresponding color readings were 54 and 56.

TABLE II

Fractionation of Starch with Water at 80°

	Yield 1	Yield 2	Intensity of iodine color*	
			Yield 1	Yield 2
	per cent	*per cent*		
Fraction I	16.0	16.3	220	224
" II	Trace	Trace		
" III	82.9	79.6	114	104

* See Table I.

TABLE III

Fractionation by Butanol Extraction

Fraction	Yield 1	Yield 2	Iodine coloration*	
			Yield 1	Yield 2
	per cent	*per cent*		
Butanol-pptd. by autoclaving	38.6	39.2	243	241
" " Waring blendor	39.5	39.4	234	230
Butanol-non-pptd. by autoclaving	60.7	59.9	52	49
" " Waring blendor	59.4	60.7	47	50

* See Table I.

Fractionation by Selective Precipitation of Amylose with Butanol—Schoch's butanol precipitation method (4) was closely followed, except for the purification of the separated amylose by recrystallization from the boiling water-butanol mixture. Since it was thought that some hydrolytic degradation of starch was likely to occur as a result of the high temperature treatment under pressure, an attempt was made to eliminate autoclaving in Schoch's procedure by securing dispersion of starch with high speed stirring. A properly gelatinized paste of 5 gm. of starch in about 500 ml. of boiling water was treated in a Waring blendor in two lots for 5 minutes each. Subsequent separation of the starch fractions was effected as described by Schoch. Typical results, by both procedures, are given in Table III.

Fractional Precipitation of Amylose with Thymol—The high speed stirring recommended by Haworth *et al.* (5) did not result in sufficient dispersion and it was found more effective to use a Waring blendor for aiding dispersion. The results obtained by this method are shown in Table IV.

Fractionation by Preferential Adsorption of Amylose on Cotton—With a 1 per cent starch paste, gelatinized and dispersed in a Waring blendor according to the procedure of Pacsu and Mullen (7), not more than 0.3 per cent of amylose (iodine coloration, average 223) was obtainable, therefore suggesting that preferential adsorption of amylose on the cotton used was far from satisfactory; somewhat similar results were obtained by using filter paper pulp as an adsorbent.

TABLE IV

Fractionation by Thymol Extraction

Fraction	Yield 1	Yield 2	Yield 3	Iodine coloration*		
				Yield 1	Yield 2	Yield 3
	per cent	*per cent*	*per cent*			
Thymol-pptd	39.7	39.9	40.7	222	220	208
Thymol-non-pptd	60.1	60.2	59.1	50	52	45

* See Table I.

DISCUSSION OF RESULTS

The pronounced difference in the affinity of amylose and amylopectin for iodine has formed the basis for the determination of their relative proportions in starches potentiometrically (8), absorptiometrically (3), or spectrophotometrically (9, 10). On the assumption that the intensity of iodine coloration with amylose or amylopectin fractions will be determined by their respective freedom from each other, it becomes apparent that the higher the scale reading, the purer will be the fraction in respect to its amylose content, and that, conversely, purity with regard to amylopectin will be connoted by the lowest scale reading. Based on this criterion, it would follow from the data presented here (Tables I to IV) that no single method effects simultaneously efficient and quantitative separation of the two starch fractions. The procedure of McCready and Hassid (Table I) gives, in one step, the purest amylose fraction, judging from its iodine-staining property; its solubility is, however, only of the order of 15 per cent, which is very low. That the amylopectin fraction obtained here is admixed with a high amount of amylose is evident from the values for iodine coloration as compared to the amylopectin fraction obtained by the procedures of Schoch (Table III) and of Haworth *et al.* (Table IV). The amylose fraction yielded by Meyer's extraction procedure (Table II) is only about 80 per cent as pure as the corresponding fraction obtained according to the method of McCready and Hassid; this is no doubt due to the fractionation temperature being near the gelatinization point of the starch and consequent contamination with amylopectin by the disintegration and rupture of the granules. The use of chloral hydrate solution to purify amylopectin from admixed amylose resulted in a product still containing about 6 to 10 per cent of amylose.

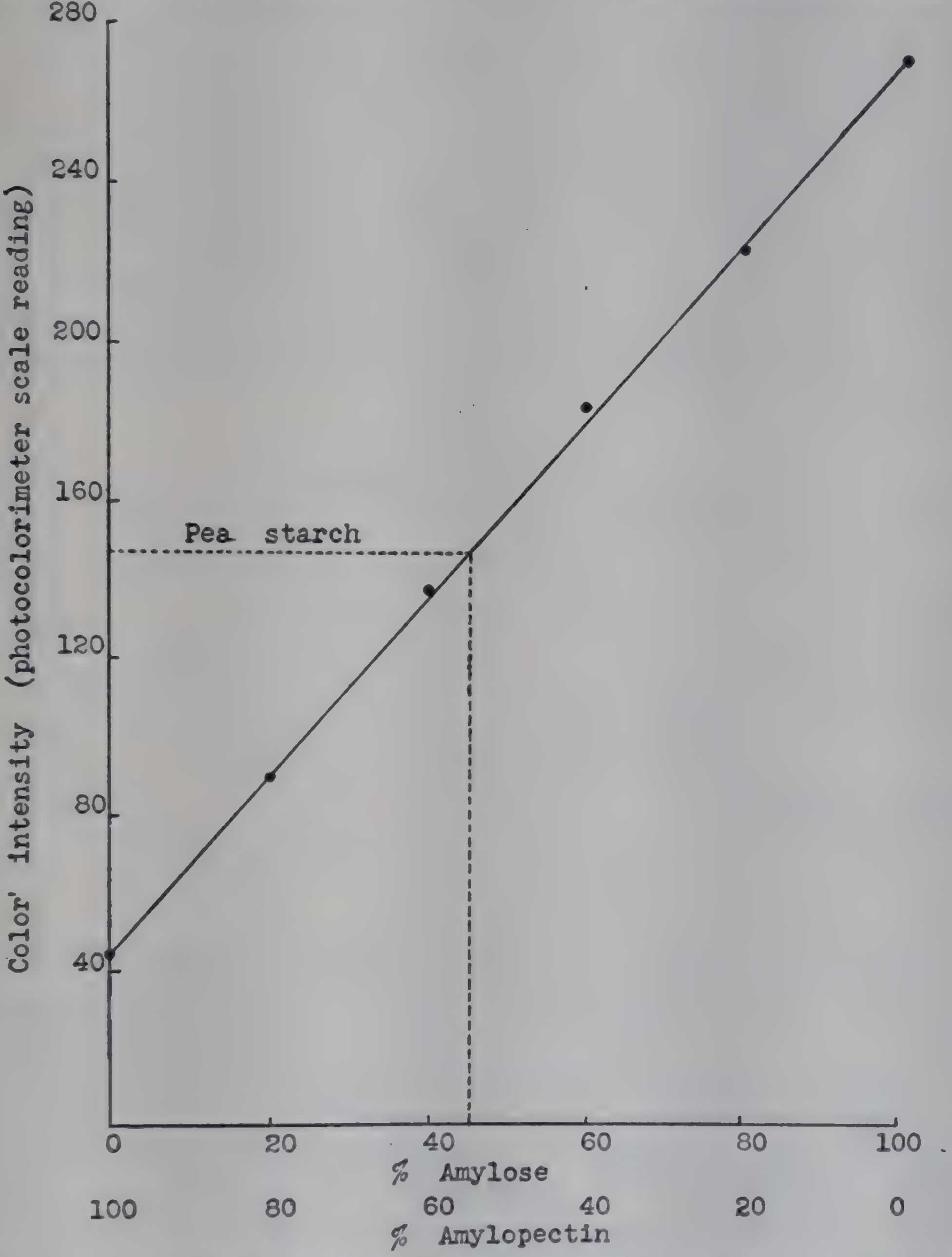


FIG. 1. Color intensities of mixtures of amylose and amylopectin from pea starch with iodine.

Both butanol and thymol undoubtedly effect very much better fractionation of the starch components than does extraction with hot water, and, indeed, the yields of amylose and amylopectin correspond more nearly to the correct values deduced below than do those obtained by hot water extraction. However, it is clear from a comparison of the iodine coloration of amylose fractions (Tables III and IV) that they are respectively only about 85 and 77 per cent as pure as that obtained by the McCready and Hassid method. It has been possible to obtain pure amylose by successive recrystallizations from boiling water-butanol mixtures as recommended by Schoch (4, 11), but, the yields being no longer quantitative, it appeared preferable to do so in a single extraction with hot water at 60°.

Although the amylopectin fractions obtained by selective precipitation with butanol or thymol have given the lowest intensity of iodine coloration of all the methods studied, it was felt that, since it is always the residue in the mother liquor after the amylose had been precipitated, amylopectin may not be easily obtainable in as pure a form as the amylose component. We therefore attempted to ascertain whether by butanol fractionation of the residue from the hot water treatment of starch at 60° (Fraction III, Table I) a purer preparation of amylopectin could be obtained than by Schoch's method from the original starch. By this procedure, a product was secured which gave an iodine coloration of only 43 or 44 units. This was the purest amylopectin obtainable; a product with similar purity could also be prepared by thymol fractionation of crude amylopectin.

TABLE V

Fractionation of Mung Starch

Starch analysis	
Moisture, %	13.50
Proteins, %	0.69
Ether extractives, %	0.23
Iodine coloration of starch*	127
" " " amylose fraction*	287
" " " amylopectin fraction*	34
Amylose (from Fig. 2), %	36.8
Amylopectin (from Fig. 2), %	63.2

* Scale readings, Klett-Summerson colorimeter.

Pure preparations of amylose and amylopectin can thus be obtained in one and two operations, respectively, by a combination of the features of McCready and Hassid's method for amylose and that of Schoch or of Haworth *et al.* for amylopectin. By using various proportions of the starch components prepared as above, the color intensities of the mixtures in solution (2 mg. per 100 ml.) with iodine can be plotted against per cent concentration of the two fractions when a linear relationship similar to that reported by McCready and Hassid (3) is obtained (Fig. 1), and from which, after ascertaining the color intensity of the original starch with iodine under identical conditions, its proportions of the two constituents can be read; the latter can also be deduced by simple extrapolation, as there is strict

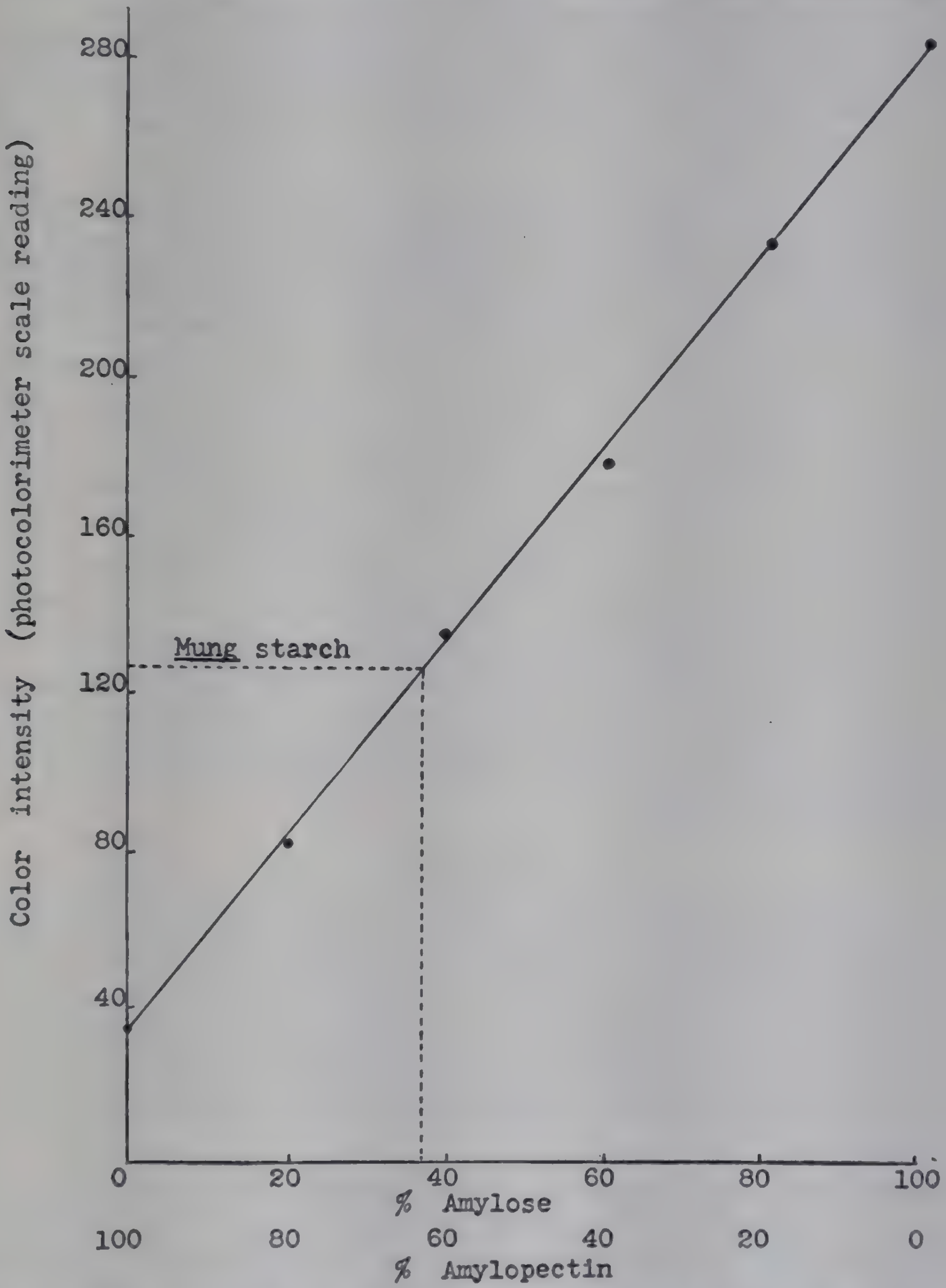


FIG. 2. Color intensities of mixtures of amylose and amylopectin from mung starch with iodine.

proportionality between color intensity with iodine and amylose or amylopectin content. In this way, the preparation of pure starch used in these studies, with its iodine coloration of 147 (Table I), can be observed to consist of 45.0 per cent amylose and 55.0 per cent amylopectin.

By the foregoing procedures for the preparation of pure amylose and amylopectin fractions, and by using a preparation of mung (*Phaseolus radiatus*) starch, the resulting observations are given in Table V and in Fig. 2.

Differences such as are recorded here in intensities of iodine coloration with pure amylose or amylopectin preparations from natural starches are bound to exist because of possible heterogeneity as to molecular size as well as, with amylopectin, to variations in the degree of branching (*cf.* (8)).

Although the various methods for the fractionation of starch examined here do not effect a clear-cut and quantitative separation of the unbranched and branched components in their pure state, fractionation by selective precipitation of amylose with butanol or with thymol, as recommended by Schoch (4) and by Haworth *et al.* (5), gives an approximate idea of the relative proportions of the two constituents. However, to obtain them in a pure state for examination of their individual properties or for a precise evaluation of their percentages in any starch sample by reference to a calibrated curve or by extrapolation, as described here, it would appear necessary to resort to a combination of procedures involving the properties of amylose for selective diffusibility in water at 60° and precipitability with butanol- or thymol-saturated water.

SUMMARY

1. A comparative study has been made of the procedures for the fractionation of starch based on the differential solubilities of amylose and amylopectin in hot water and in butanol- or thymol-saturated water.

2. It is shown that the method of extraction with hot water at 60° yields an amylose fraction which is the purest obtainable, judged from the intensity of its coloration with iodine; amylose separation is not, however, quantitative.

3. Fractionation of starch by selective precipitation of amylose with butanol or thymol gives only a rough indication of the relative proportions of the linear and branched components; besides, separation, as judged by the iodine-staining properties of the products obtained, is not clear-cut.

4. A procedure is outlined for obtaining highly pure preparations of amylose and amylopectin from a starch sample. By quantitatively determining the color intensities of the starch and of known mixtures of its amylose and amylopectin fractions with iodine, their proportions in the former can be precisely estimated.

BIBLIOGRAPHY

1. Meyer, K. H., in Kraemer, E. O., Advances in colloid science, New York **1**, 143 (1942).
2. Peat, S., in Annual review of biochemistry, Stanford University, **15**, 75 (1946).
3. McCready, R. M., and Hassid, W. Z., *J. Am. Chem. Soc.*, **65**, 1154 (1943).
4. Schoch, T. J., *J. Am. Chem. Soc.*, **64**, 2957 (1942).
5. Haworth, W. N., Peat, S., and Sagrott, P. E., *Nature*, **157**, 19 (1946).
6. Whistler, R. L., and Hilbert, G. E., *J. Am. Chem. Soc.*, **67**, 1161 (1945).
7. Pacsu, E., and Mullen, J. W., 2nd, *J. Am. Chem. Soc.*, **63**, 1168 (1941).
8. Bates, F. L., French, D., and Rundle, R. E., *J. Am. Chem. Soc.*, **65**, 142 (1943).
9. Kerr, R. W., and Trubell, O. R., *Paper Trade J.*, **117**, No. 15, 25 (1940).
10. Baldwin, R. R., Bear, R. S., and Rundle, R. E., *J. Am. Chem. Soc.*, **66**, 111 (1944).
11. Wilson, E. J., Jr., Schoch, T. J., and Hudson, C. S., *J. Am. Chem. Soc.*, **65**, 1380 (1943).

THE INCORPORATION OF ACETATE AND BUTYRATE CARBON INTO RAT LIVER GLYCOGEN BY PATHWAYS OTHER THAN CARBON DIOXIDE FIXATION*

By NATHAN LIFSON, VICTOR LORBER, WARWICK SAKAMI, AND HARLAND G. WOOD

(From the Department of Physiology, University of Minnesota, Minneapolis, and the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland)

(Received for publication, July 23, 1948)

This investigation was undertaken to study, by use of acetic and butyric acids labeled with C^{13}, the conversion in the intact animal of lower fatty acid carbon to liver glycogen in order to help explain the intermediate reactions linking fat and carbohydrate metabolism in the intact animal. It appears certain that such conversion does occur for both of these acids (1).

Classically, a dietary constituent has been considered a glycogen former if, after its administration, a net increase in glycogen is found over and above that of the control. By this criterion, there is no general agreement whether acetate and butyrate are glycogen formers. However, the carbon of a fatty acid could enter liver glycogen without effecting a net increase in the glycogen. For example, carbon could enter because of the constant turnover between carbohydrate and its precursors. Furthermore, because of the influence of the dynamic equilibrium between fats, proteins, and carbohydrates there could actually be a decrease in glycogen after administration of a fatty acid and still carbon of the fatty acid could enter the latter. Similarly, a net increase in glycogen could conceivably occur without transfer of carbon from the fatty acid to glycogen. The question of whether acetate and butyrate are liver glycogen formers in the classical sense is different from that being considered in the present work; namely, whether the carbon of these fatty acids is incorporated into liver glycogen.

The fatty acids in question are relatively rapidly metabolized to CO_2 (1), and CO_2 itself can be incorporated into liver glycogen (2). The biochemical significance of the presence of fatty acid carbon in glycogen therefore depends to a considerable degree upon (*a*) whether all such carbon entered glycogen via CO_2 fixation or (*b*) whether some entered by another route without first being converted to CO_2.

It is possible to degrade the glucose units of liver glycogen to locate the

---

* This work was supported in part by grants from the Graduate School of the University of Minnesota and from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

position of the labeled carbon (2). In the case of CO_2 fixation, the isotopic carbon has been detected only in positions 3 and 4 of the glucose (2).[1] Should the heavy carbon in glycogen isolated after feeding isotopic fatty acid appear in positions other than 3 and 4, this would be rather convincing evidence that a pathway other than CO_2 fixation was involved. In the experiments to be reported, the distribution of C^{13} has been determined in rat liver glycogen after the administration, respectively, of each of the following acids: $CH_3C^{13}OOH$, $C^{13}H_3COOH$, $C^{13}H_3C^{13}OOH$, $CH_3CH_2CH_2C^{13}OOH$, $CH_3CH_2C^{13}H_2COOH$, $CH_3C^{13}H_2CH_2COOH$. Evidence has been obtained which indicates that these acids are converted to glycogen by another route in addition to CO_2 fixation, and some indications have been obtained as to the mechanisms of these conversions. Preliminary reports of this work have been published previously (3–5).

Methods

Preparation of Isotopic Fatty Acids—The carboxyl-labeled fatty acids were prepared by the Grignard reaction from the appropriate bromide and isotopic CO_2. Purity of these acids was checked by determination of titratable acidity, total carbon, and partition coefficients (6); all agreed with theoretical values except the partition coefficient of the $CH_3 \cdot CH_2 \cdot CH_2C^{13}OOH$, which was 30.6 as compared with 31.1 for known butyric acid.

The $C^{13}H_3COOH$ was synthesized by a modification of the method of Anker (7). The purity of this and the following compounds was established by the same procedures that were used with the carboxyl-labeled acids. Agreement with theory was satisfactory unless otherwise indicated.

The $C^{13}H_3 \cdot C^{13}OOH$ was synthesized by a procedure which was based upon the method of Cramer and Kistiakowsky (8). In the modification 50 mM of $BaC^{13}O_3$ were used. The acetylene-hydrogen mixture as formed on hydrolysis of the barium carbide magnesium was collected over water in a 3 liter flask and was then passed through a sintered glass disk into 150 ml. of boiling catalyzing solution. The resulting aldehyde was collected in 50 ml. of 5 per cent sodium bisulfite. The outgoing gas was collected and again passed through the catalyzing solution. The aldehyde was distilled from the bisulfite, after addition of calcium carbonate, and was oxidized to acetic acid by the dichromate method of Stahly *et al.* (9).

Butyric acid labeled in the α position with C^{13} was prepared by the following series of reactions. Carboxyl-labeled sodium propionate was synthesized by the Grignard reaction and converted into the ethyl ester by heating with diethyl sulfate. The procedure was similar to the synthesis of carboxyl-labeled ethyl acetate (10). The ethyl propionate was then hydrogenated at 250° and 220 atmospheres by use of the copper-barium-chromium oxide catalyst (11, 12). The mixture of ethyl and propyl alcohols was converted into a mixture of the corresponding iodides by heating with constant boiling hydriodic acid (13). The iodides were separated by fractionation. This separation was not complete and there was some non-labeled ethyl iodide in the propyl iodide fraction. The isotopic propyl iodide was converted into α-labeled butyric acid by the Grignard reaction. The over-all yield was 22 per cent of the theory. From 60 mM of isotopic $BaCO_3$, as determined by the partition coefficient, 13.1 mM of α-labeled butyric acid were obtained, mixed with 5.3 mM of non-isotopic propionate.

---

[1] In experiments carried out subsequently with $C^{14}O_2$, it has been found that actually a trace of isotope does appear in carbon atoms 1, 2, 5, and 6. The specific activity of these positions has been found to be 1 to 2 per cent of that of carbon atoms 3 and 4.

Butyric acid labeled in the β position with C^{13} was synthesized by the following procedure. Carboxyl-labeled acetic acid was esterified with *n*-butyl alcohol and hydrogenated at 250° and 220 atmospheres with the copper-barium-chromium oxide catalyst. The mixture of ethyl and butyl alcohols was converted into the corresponding mixture of iodides and fractionated. The isotopic ethyl iodide was converted into butyric acid by the malonic ester procedure (14). The butyric acid and some non-labeled acetic acid from the malonic ester were converted into the methyl esters by heating the potassium salts with dimethyl sulfate and purified by fractionation. The methyl butyrate was saponified, and the butyric acid identified by determination of the partition coefficient. The over-all yield was 25 per cent. From 93.5 mM of sodium acetate 24.5 mM of β-labeled butyric acid mixed with 0.96 mM of non-isotopic acetic acid were obtained.

Before administration to the experimental animals, the solution of the sodium salt was made just acid to phenolphthalein by the addition of HCl.

General Experimental Procedure—The procedure of Buchanan *et al.* (1) has been adopted, in which glucose is fed to fasted rats, together with the isotopic fatty acid. Under such conditions a net deposition of isotope-containing glycogen occurs and there is relatively minor dilution of the newly formed glycogen by preformed non-isotopic glycogen.

Table I contains data concerning the animals used, the material administered, the liver weight, and the quantity of liver glycogen recovered.

Rats, 120 to 240 gm. in weight, raised on a diet of Promax rat food,[2] were fasted for approximately 24 hours. Immediately after their teeth were clipped, the rats were fed by stomach tube a solution containing 1.7 to 3.3

---

[2] Analysis by manufacturer, in per cent, protein 29.5, fat 8.40, fiber 2.8, nitrogen-free extract 41.45, moisture 9.00, ash 8.85, calcium 1.20, phosphorus 1.13.

mM of fatty acid and 353 to 477 mg. of glucose per 100 gm. of body weight. The total volume administered was usually such as to make the glucose concentration 20 per cent.

TABLE I

General Experimental Data

Rat No.	Weight after fast	Sex	Duration of fast	Fatty acid administered	Amount administered	Glucose administered	Liver weight	Liver glycogen (as glucose)
	gm.		*hrs.*		*mM per 100 gm. body weight*	*mg. per 100 gm. body weight*	*gm.*	*mg.*
33	234	♂	24	$\mathrm{CH_3C^{13}OOH}$	2.5	400	8.4	29
34	215	♂	24	"	2.5	400	7.6	
38	215	♀	50	"	2.5	400	5.8	81
100	155	♀	24	$\mathrm{C^{13}H_3COOH}$	2.4	390	5.5	188
101	148	♀	24	"	2.4	400	5.8	
102	153	♀	24	"	2.4	400	5.8	116
103	165	♀	24	"	2.5	400	5.5	
51	190	♂	24	$\mathrm{C^{13}H_3C^{13}OOH}$	2.9	420	5.6	35
63	210	♂	27	"	2.7	410	7.5	100
64	210	♂	27	"	2.7	410	7.4	85
69	200	♀	24	"	2.8	450	5.9	500
70	200	♀	24	"	2.8	450	5.2	
71	183	♀	24	"	2.8	438	6.7	
72	179	♀	24	"	2.8	438	6.3	
73	125	♂	24	"	2.3	400	4.9	
74	135	♂	24	"	2.3	400	5.5	
75	140	♂	24	"	2.3	400	5.5	
53	240	♂	24	$\mathrm{CH_3CH_2CH_2C^{13}OOH}$	2.6	417	8.3	115
67	230	♂	24	"	2.5	400	6.7	90
81	140	♂	24	$\mathrm{CH_3CH_2C^{13}H_2COOH}$	1.82*	408	6.3	140
82	155	♂	24	"	1.79*	402	6.2	378
83	160	♂	24	"	1.81*	406	6.6	
84	140	♂	24	"	1.74*	390	6.1	
89	140	♂	24	$\mathrm{CH_3C^{13}H_2CH_2COOH}$	2.5	400	6.1	129
90	130	♂	24	"	2.5	400	6.2	96
91	130	♂	24	"	2.5	400	5.5	91
92	130	♂	24	"	2.5	400	5.8	105

* The solution contained appreciable amounts of non-isotopic propionate.

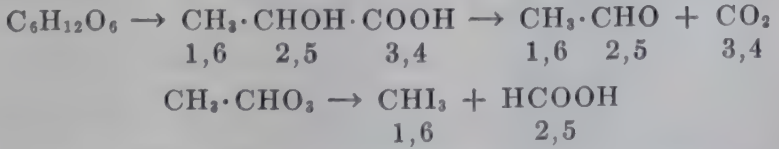
The animals were placed in a metabolism chamber for collection of their respiratory CO_2 in NaOH, as previously described (2), with the modification that in the experiments other than those with carboxyl-labeled acids both the alkali and air were stirred. There were three collection periods of 50 minutes each, between which the chamber was opened for changing the alkali, a series of manipulations requiring less than a minute in nearly all cases. The CO_2 content of a sample of the air of the chamber taken at the end of each of two collection periods was found to be 0.16 and 0.18 per cent, respectively. Since the total volume of the chamber was about 11 liters, it may be estimated that some 20 ml. of CO_2 remained uncollected per period or less than 3 mM for an entire experiment. The CO_2 content of the alkali was measured by analyzing an aliquot in the manometric Van Slyke apparatus.

Usually 2½ hours after feeding the fatty acid-glucose solution, the rats were anesthetized with sodium amytal (100 mg. per kilo, intraperitoneally). The liver was extirpated, immediately placed in hot 30 per cent KOH, and heated in a boiling water bath for 3 hours.

Glycogen was isolated by a modification of the method of Stetten and Boxer (15) in all experiments but those with the carboxyl-labeled acids, in which the procedure of Good, Kramer, and Somogyi (16) was used. As previously noted (2), when the procedure of Good *et al.* was used, the total carbon, as determined by a modification of the Van Slyke and Folch technique (17), amounted to 117 to 184 per cent of the glucose estimated by reducing power. When the procedure of Stetten and Boxer was employed, the corresponding values were 94 to 112 per cent.

The glycogen was converted to glucose by hydrolysis in H_2SO_4. To remove any volatile C^{13} fatty acid, the hydrolysis was begun in 0.25 N acid, which was concentrated in a boiling water bath to 1 N by evaporation to one-fourth the original volume. The original volume was restored with water and the concentration repeated three times, whereupon the hydrolysis was completed in the remaining 1 N acid. The hydrolysate was decolorized with charcoal and filtered. Glucose was determined by either the Shaffer-Hartmann method (18) or the method of Folin and Malmros (19).

Two types of degradation of glucose, bacterial and chemical, were carried out as previously described (2). In the bacterial degradation the glucose is fermented to lactic acid and then the lactic acid is oxidized with $KMnO_4$ to acetaldehyde and CO_2. The acetaldehyde is in turn converted by the iodoform reaction to iodoform and formic acid. In this manner the carbons of the glucose are obtained in three fractions containing respectively carbon atoms 3 and 4, 2 and 5, and 1 and 6.



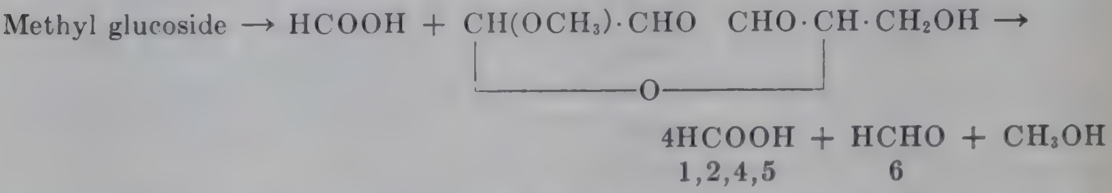
$$C_6H_{12}O_6 \longrightarrow \underset{1,6}{CH_3}\cdot\underset{2,5}{CHOH}\cdot\underset{3,4}{COOH} \longrightarrow \underset{1,6}{CH_3}\cdot\underset{2,5}{CHO} + \underset{3,4}{CO_2}$$

$$CH_3\cdot CHO_3 \longrightarrow \underset{1,6}{CHI_3} + \underset{2,5}{HCOOH}$$

In several instances the acetaldehyde was oxidized directly with persulfate (20) instead of being subjected to the iodoform reaction; thus carbon atoms

1, 2, 5, and 6 were obtained in one fraction. Ether extraction of the lactic acid was performed only in experiments on Rats 69 to 75 and Rats 100 to 103; in other instances the oxidation was carried out directly on the centrifuged solution from the bacterial fermentation.

In the chemical degradation, with limitations as described previously (2) and in this communication, carbon atoms 3 and 6 are obtained as individual fractions and carbon atoms 1, 2, 4, and 5 as a third fraction.



$$\text{Methyl glucoside} \rightarrow \text{HCOOH} + \text{CH(OCH}_3\text{)}\cdot\text{CHO} \quad \text{CHO}\cdot\text{CH}\cdot\text{CH}_2\text{OH} \rightarrow$$

$$\underset{1,2,4,5}{4\text{HCOOH}} + \underset{6}{\text{HCHO}} + \text{CH}_3\text{OH}$$

Heavy carbon was determined with the mass spectrometer. The values are expressed as atom per cent excess C^{13}, *i.e.* C^{13} in excess of normal (unenriched) carbon, commercial reagent grade $BaCO_3$ being employed as a source of a CO_2 standard in calibrating the mass spectrometer. The maximum difference in C^{13} content between this standard and normal rat liver glycogen was found to be 0.005 atom per cent. Whereas this result might indicate 0.005 to be the expected experimental variation, it has been our procedure not to place significance on values less than 0.02 atom per cent in excess of normal. Thus a margin is provided for possible incomplete separation of the isotopic and non-isotopic compounds.

In a control experiment non-isotopic acetate was administered to the animal, but at the time the liver was placed in KOH, carboxyl-labeled acetate was also added to the alkali. Significant amounts of excess C^{13} were not found in the glycogen (see Table II, results for Rat 35).

RESULTS AND DISCUSSION

The discussion will take the following order. First the data on the relative distribution of the isotope in the glucose unit of the glycogen will be considered for each of the different types of labeled acids. Next evidence will be presented which shows that these acids are converted to glycogen by a pathway in addition to that of CO_2 fixation. Finally the intermediary mechanism of metabolism of acetate and butyrate will be considered in relation to the tricarboxylic acid cycle and glycogen synthesis.

Distribution of Isotope in Glucose from Liver Glycogen

The distribution of C^{13} in the liver glycogen following the administration of labeled fatty acids is shown in Table II; the values are from both the bacterial and chemical degradations. Although there are some discrepancies between results by the two methods, which will be considered later,

TABLE II

Distribution of C^{13} in Liver Glycogen after Administration of Labeled Fatty Acids

C^{13} values are expressed in terms of atom per cent excess.

Fatty acid administered	C^{13} whole fatty acid molecule	Rat No.	C^{13} in bacterial degradation fractions			C^{13} in chemical degradation fractions			C^{13} whole glucose molecule	Location of isotope in glycogen
			Carbon atoms of glucose			Carbon atoms of glucose				
			3,4	2,5	1,6	3	1,2,4,5	6		
$CH_3C^{12}OOH$	0.00	35	0.015	0.01						C—C—C—C—C—C
$CH_3C^{13}OOH$	2.63	33, 34	0.10	0.01						C—C—C*—C*—C—C
$C^{13}H_3COOH$	1.98	100–103	0.08	0.18	0.16†	0.10	0.17	0.19	0.14	C*—C*—C^0—C^0—C*—C*
$C^{13}H_3C^{13}OOH$	4.30	51	0.24	0.19	0.14‡					C*—C*—C*—C*—C*—C*
"	4.30	63, 64				0.29	0.25	0.23	0.26	
"	4.60	69–75	0.36	0.29	0.28§	0.32	0.28	0.24	0.29	
$CH_3CH_2CH_2C^{13}OOH$	0.98	53	0.13	0.01	0.01					C—C—C*—C*—C—C
"	0.98	67	0.18	0.02	0.01					
$CH_3CH_2C^{13}H_2COOH$	0.97	81	0.04	0.17	0.14‖					C*—C*—C^0—C^0—C*—C*
"	0.97	82–84	0.05	0.16	0.14¶	0.05	0.14	0.17	0.14	
$CH_3C^{13}H_2CH_2COOH$	1.09	90	0.12	0.02	0.01					C—C—C*—C*—C—C
"	1.09	89, 91, 92	0.16	0.02	0.02	0.11	0.05	0.01	0.06	

Mg. of CO_2 in sample (†) 28.5, (‡) 8.2, (§) 19.8, (‖) 15.0, (¶) 14.9.

the over-all results can be summarized from the data as given. It is seen that with both carboxyl-labeled acetic and butyric acids glucose was obtained in which the isotope was predominantly in the 3 and 4 positions. There was little or no excess in the 2,5 and 1,6 positions. This fact has been indicated in the last column of Table II by means of a 6-carbon skeleton (C—C—C*—C*—C—C) in which the asterisk locates the preponderance of isotope. A similar glucose is obtained with the β-labeled butyrate. On the other hand, with methyl-labeled and doubly labeled acetate and with α-labeled butyrate, a significant concentration of isotope was found in all degradation fractions and the indications are that each position of the glucose contained isotope. In the case of the methyl-labeled acetate and α-labeled butyrate the 3 and 4 positions contained a *smaller* but significant excess of isotope than the other positions, and this type of glucose has been indicated as follows: $C^*—C^*—C^0—C^0—C^*—C^*$. With the doubly labeled acetate all positions were high, but the 3 and 4 positions were the highest.

Before further consideration is given to the significance of these data, the reliability of the degradation procedures will be discussed. It will be noted in Table II that whenever there was an excess of isotope in the 2,5 and 1,6 positions as obtained by the bacterial degradation the concentrations were usually of the same order of magnitude in the two fractions, but frequently the 1 and 6 positions contained somewhat less excess C^{13} than did the 2 and 5 positions. It was of considerable importance to establish whether or not these small differences were true differences, for, as will be shown in the later discussion, an unequal concentration would not be compatible with the currently accepted schemes of carbohydrate metabolism.

It has been found that the observed values are not true ones and that an error is caused by traces of extraneous carbon which are oxidized to CO_2 by the chromic acid oxidation used to convert the iodoform carbon to CO_2. Dilution from this source may be appreciable when samples of the size usually dealt with in the present work are involved (0.5 mM of CO_2 and less). In the case of the 2,5 position when mercuric acetate is used to convert the formic acid to CO_2, no comparable dilution occurs.

Some of the evidence on this point follows: When CO_2 was liberated from $BaC^{13}O_3$ by the chromic acid oxidant, it regularly displayed a lower C^{13} concentration than the original $BaC^{13}O_3$, unless enough $BaC^{13}O_3$ was used to obviate the effects of dilution by the extraneous carbon which was oxidized to CO_2. The same type of dilution was observed when the chromic acid oxidation was applied to a labeled organic compound ($C^{13}H_3CH_2COOH$). The resulting CO_2 contained a progressively greater C^{13} concentration as the sample size was increased (from 5 to 40 mg. of CO_2). When lactic acid, which was synthesized chemically (8) from isotopic acetylene, and thus contained an equal concentration of C^{13} in the α- and β-carbons, was degraded in the usual way, a discrepancy was noted between the labeled positions similar to that found with the lactate from liver glycogen. With a large enough sample the C^{13} content of the CO_2 derived from the α- and β-carbons of the synthetic lactate was found to be the same. These results are summarized in Table III. In addition when two aliquots of the lactate from the liver glycogen of Rats 69 to 75 were degraded, the smaller samples showed a discrepancy between the C^{13} content of the α- and β-carbons, whereas the larger samples contained practically identical isotope concentrations (Table III). It is thus clear that with small samples false and variably low values were obtained for the 1 and 6 positions.

The results indicate with reasonable certainty that there is no difference between the 2,5 and 1,6 positions, and that any difference shown in Table II may be the result of the small samples employed. The present results are therefore considered to be in agreement with the idea that the 2,5 and 1,6 positions contained equal concentrations of isotope.

TABLE III

Effect of Sample Size on Apparent Isotope Content of α- and β-Carbon Atoms of Labeled Lactate

Material degraded	α-Carbon		β-Carbon	
	CO_2	C^{13}	CO_2	C^{13}
	mg.	*atom per cent excess*	*mg.*	*atom per cent excess*
Synthetic $C^{13}H_3C^{13}HOHCOOH$	10.6	3.32	10.6	2.62
" "	21.1	3.37	18.1	2.96
" " *	24.9	1.14	31.5	1.16
Aliquot of lactate from Rats 69–75	8.3	0.28	12.5	0.22
" " " " " 69–75	18.2	0.29	19.8	0.28

* Run on a sample of lactate containing less isotope than that used for the first two degradations.

It should be pointed out that the degradation cleanly separates the different positions of the lactic acid. Thus in the degradation of synthetic $C^{13}H_3C^{13}HOHCOOH$, the CO_2 isolated from the carboxyl group contained no excess C^{13} and the α- and β-carbon fractions contained equal C^{13} concentrations. On the other hand when a sample of synthetic $CH_3C^{13}HOHCOOH$ was degraded, it yielded excess isotope only in the single fraction corresponding to the α-carbon atom.

The results from the chemical degradation will be considered next. The data from this degradation in conjunction with the data from the bacterial degradation provide an opportunity to determine in greater detail the distribution of the isotope in the sugar.

Sufficient material was available in four of the glucose samples for both a bacterial and a chemical degradation. In the chemical degradation, probably the most reliable result is for position 6, since the formation of formaldehyde in the periodic acid oxidation is quite specific for primary alcohols. It will be noted in Table II that position 6 as determined by chemical degradation contained approximately the same concentration of isotope as did the 1,6 and 2,5 fractions obtained from bacterial degradation. The only exception is the result from the doubly labeled acetate, Rats 69 to 75, in which the concentration of isotope in position 6 was 0.24 as compared to that of 0.29 and 0.28 for the 2,5 and 1,6 positions. Possibly this result may be in error. A discrepancy in the results is indicated, because the average concentration of all fractions by bacterial degradation was higher than those from chemical degradation.

On the whole, however, it is seen that the results are in agreement with the idea that carbon atoms 1, 2, 5, and 6 contain an equal concentration of isotope. This follows from the observation that in general carbon atom 6 = 1,6; therefore carbon atom 1 = 6; also 1 or 6 or 1,6 = 2,5. It is to be noted that, although the results offer direct data indicating equality of positions 1 and 6, they do not give direct evidence that position 2 equals position 5. It seems likely, however, that this is the case from a consideration of the schemes of glycolysis.

An evaluation of the relative concentration of C^{13} in positions 3 and 4 may be obtained from the value for position 3 as determined chemically and that of the value for combined positions 3 and 4 from bacterial degradation. Only a rough approximation is possible, however, because position 3 in the chemical degradation is not completely specific. Position 3 as obtained in this method consists of formic acid and it is contaminated by formic acid arising from other positions. In part this contamination comes from unmethylated glucose, since the samples of glucoside contained at least 1 to 2 per cent free glucose. In addition some free glucose may be formed by hydrolysis of the glucoside during the oxidation. In the periodic acid oxidation of free glucose formic acid is formed from 5 of the 6 carbon atoms. The fact that contamination does occur is indicated by results obtained on glucoside prepared from pure glucose in which more than a mole for mole yield of formic acid was found (2). Contamination of position 3 becomes apparent when the 1,6 and 2,5 positions have a low or high concentration of isotope as compared to the 3 and 4 positions. Thus when 1, 2, 5, and 6 carbon atoms contained more isotope than carbons 3 and 4, the contamination gave a high value for carbon atom 3 as compared to carbons 3 and 4; on the other hand when carbon atoms 1, 2, 5, and 6 contained a low concentration of isotope, the opposite effect occurred. These results were noted in most cases (see Table II).

When consideration is taken of these sources of contamination of position 3, comparison of position 3 from chemical degradation with positions 3 and 4 from bacterial degradation indicates that position 3 is approximately equal to positions 3 and 4. Thus carbon atoms 3 and 4 probably contain an equal concentration of C^{13}. All the results of Table II, therefore, are in conformity with the idea that the two halves of glucose are identical. This is the distribution of isotope which is to be expected on the basis of the conventional schemes of glycolysis in which glucose is formed on an over-all basis by union of 2 pyruvate molecules through a carboxyl to carboxyl linkage (2). This prediction assumes that the triose isomerase acts sufficiently rapidly to equilibrate the isotope in the glyceraldehyde phosphate and dihydroxyacetone phosphate. It is apparent, however, that the methods do not permit the strictly quantitative measurement which would be necessary to prove this point rigidly.

Evidence for Conversion of Fatty Acid Carbon to Glycogen by Pathway Other Than CO_2 Fixation

It is apparent from Table II that after the feeding of carboxyl-labeled acetate or butyrate, as well as of β-labeled butyrate, all, or practically all, of the detectable excess C^{13} was found in positions 3 and 4 of the glucose. Since these are the same positions as for CO_2 fixation, the results with these compounds provide no direct evidence that there is a mechanism other than CO_2 fixation by which the carbon of acetic or butyric acid is incorporated into liver glycogen. However, after the feeding of α-labeled acetate, doubly labeled acetate, or of α-labeled butyrate, not only carbon atoms 3 and 4, but all degradation fractions contained significant amounts of C^{13}. These latter results furnish direct evidence that at least the α-carbon atoms of the fatty acids studied are incorporated into liver glycogen by some means in addition to CO_2 fixation. The possiblity that the presence of fatty acid has provided a route over which carbon may be incorporated as CO_2 into positions other than 3 and 4 of the glucose is excluded by the results obtained when the carboxyl-labeled acids were fed.

The level of isotope in the respiratory CO_2 has been used in attempting to evaluate the extent to which isotope from administered fatty acids reaches liver glycogen by CO_2 fixation. Buchanan *et al.* (1) fed rats acetate, propionate, and butyrate labeled with C^{11} in the carboxyl position. On the basis of the radioactivity found in the liver glycogen and respiratory CO_2 in these experiments as compared with the radioactivity found in liver glycogen and respiratory CO_2 in CO_2 fixation experiments, they attempted to apply a correction to the fatty acid experiments for the isotope deposited in the liver glycogen from CO_2. An observed isotope content in the glycogen over and above that calculated as derived from CO_2 was taken to indicate conversion of fatty acid to glycogen by a second mechanism. These workers concluded that in the case of acetate CO_2 fixation alone could account for all of the C^{11} of the glycogen, whereas for propionate and butyrate CO_2 fixation could account for only a fraction of the isotope found.

In the present work, in the case of acetate as well as of butyrate, for all types of labeling employed, the isotope was more abundant in carbon atoms 3 and 4 of the glucose than would be anticipated from the level of C^{13} in the respiratory CO_2 were CO_2 fixation the only mechanism at work. For example, the excess C^{13} in carbon atoms 3 and 4 of the glucose isolated from the liver of rats administered $NaHC^{13}O_3$ was only about one-sixth to one-seventh that found in the respiratory CO_2 (2), while in the experiments with carboxyl-labeled acetate, the isotope in positions 3 and 4 was relatively twice as abundant as in the CO_2 fixation experiments, being about one-third the excess noted in the respiratory CO_2.

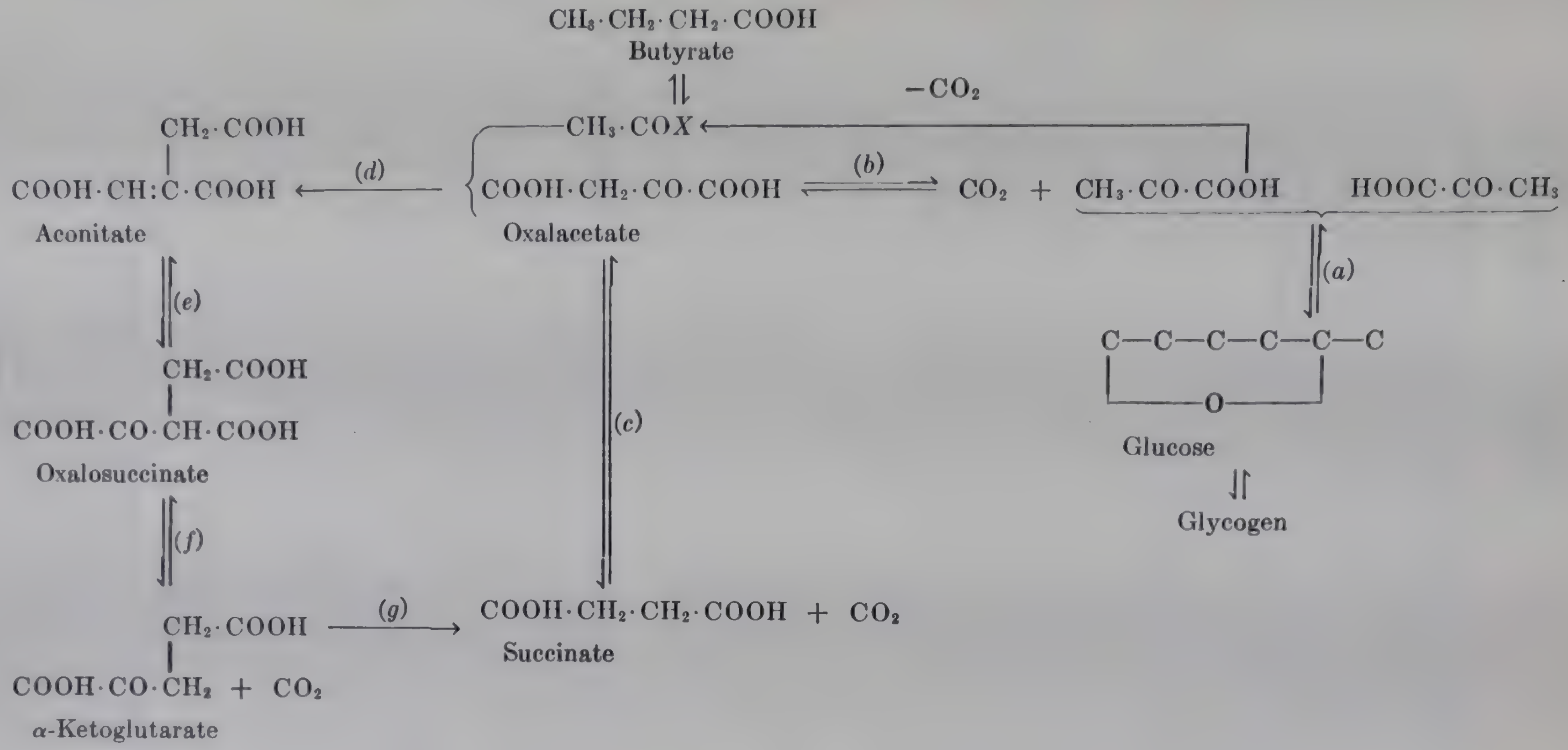
Certain objections to comparisons of this sort may be pointed out. Experiments on CO_2 fixation in which labeled bicarbonate has been given may not be regarded as suitable controls for experiments in which fatty acids have been fed, since the influence of fatty acid feeding on CO_2 fixation has not been assessed. Furthermore, the isotope content of the respiratory CO_2 may not bear the same relationship to the isotope content of the intracellular CO_2 in both instances, since the isotopic CO_2 has been introduced into the animal as bicarbonate in one case and has arisen from the intracellular oxidation of an organic acid in the other. The experiments of Ball *et al.* (21), in which $NaHC^{11}O_3$ was administered and the specific activity of the $NaHCO_3$ of pancreatic juice and of the respiratory CO_2 was found to be the same, cannot be considered as establishing the respiratory CO_2 as a valid sample of the intracellular CO_2 of all tissue cells with respect to isotope content, since the cells of external secretion of the pancreas are probably unique in their ability to concentrate bicarbonate. Hence it is apparent that experiments with the carboxyl-labeled acids provide only indirect evidence regarding the transfer of the fatty acid carbon to glycogen by ways other than CO_2 fixation, and, in view of the possible source of error described above, it is not surprising that the evidence of Buchanan *et al.* (1) and of the present work is in conflict with respect to acetate.

The present results with α-labeled and doubly labeled acetate, in which the isotope occurs in positions other than 3 and 4 of liver glucose, show conclusively, however, that this acid is converted to carbohydrate by pathways other than CO_2 fixation.

Intermediary Metabolic Steps in Conversion of Fatty Acid to Glycogen

Although in the present work we are observing only the initial and final stages whereby fatty acid carbon is transformed to glycogen, some discussion seems desirable concerning intermediate steps.

Abridged Tricarboxylic Acid Cycle and Its Relationship to Glycogen Synthesis


CH₃·CH₂·CH₂·COOH
Butyrate
−CO₂
CH₃·COX
CH₂·COOH
COOH·CH:C·COOH
(d)
COOH·CH₂·CO·COOH
(b)
CO₂ + CH₃·CO·COOH
HOOC·CO·CH₃
Aconitate
Oxalacetate
(a)
(e)
CH₂·COOH
COOH·CO·CH·COOH
(c)
C—C—C—C—C—C
O
Glucose
Oxalosuccinate
Glycogen
(f)
CH₂·COOH
(g)
COOH·CH₂·CH₂·COOH + CO₂
Succinate
COOH·CO·CH₂ + CO₂
α-Ketoglutarate

Because of the impressive evidence accumulating in favor of the tricarboxylic acid, or Krebs cycle, as a general mechanism for the oxidation of carbohydrates, fats, and proteins, the present results will be considered in terms of this cycle. The cycle, abridged for purposes of simplicity, is presented schematically in the accompanying diagram. The assumptions are made that the constituent hexose units of glycogen are formed from two 3-carbon fragments (represented by pyruvate) in accordance with the conventional schemes of glycolysis, and that the tricarboxylic acid cycle participates in the introduction of isotope into the 3-carbon fragment, pyruvate.

It must be emphasized that the following discussion of metabolic pathways for acetic and butyric acids is speculative. The possibility exists that the results of the present experiments, as well as of those on CO_2 fixation (2), may be explained by unknown metabolic conversions, or by modifications of reactions in the tricarboxylic acid cycle, or by reactions on the path between pyruvate and glucose.

In the diagram it is to be noted that the carboxyl carbon of pyruvate becomes the 3 and 4 carbons of the glucose, the α-carbon becomes the 2 and 5 carbons, and the β-carbon becomes the 1 and 6 carbons (Reaction *a*). Furthermore, since pyruvate is formed from the oxalacetate (Reaction *b*) by loss of the carboxyl adjacent to the methylene group (β-carboxyl group), the location of C^{13} in the oxalacetate determines the location of the isotope in the pyruvate.

The oxalacetate formed from pyruvate and isotopic carbon dioxide would contain labeled carbon in the β-carboxyl group only. In order to explain the location of fixed carbon dioxide in positions 3 and 4 of glycogen, it is assumed that the oxalacetate is converted to a symmetrical C_4-dicarboxylic acid, represented by succinic acid in the scheme (Reaction *c*). In this way the labeled carbon becomes randomized between both carboxyl groups. Reversal of Reactions *c* and *b* would then yield carboxyl-labeled pyruvate and hence glycogen labeled in positions 3 and 4.

Pathways from Acetate to Glycogen—From carboxyl-labeled acetate the isotope is transferred via the cycle to both carboxyl groups of the oxalacetate (Reactions *d*, *e*, *f*, *g*, *c*) and decarboxylation of the oxalacetate results in carboxyl-labeled pyruvate (Reaction *b*). Moreover, reentry of this labeled oxalacetate into the cycle cannot introduce the C^{13} into any other position in the oxalacetate, because both carboxyl groups of the oxalacetate are lost as CO_2 in the transformations of the cycle. This condition also applies in the case of CO_2 fixation. The scheme is thus in agreement with the observed results of Table II in that it indicates that either fixed carbon dioxide or the carboxyl of acetate will be only in the 3 and 4 positions of the glucose.

The results following the feeding of α-labeled acetate are also explained.

Condensation of $C^{13}H_3COOH$ with oxalacetate would lead through the cycle to the formation of oxalacetate, the non-carboxyl carbons of which are labeled. Decarboxylation of such oxalacetate yields α,β-labeled pyruvate and, hence, glycogen labeled equally in carbon atoms 1, 2, 5, and 6. These carbon atoms of the glycogen were found to contain excess isotope, but it will be observed in Table II that carbon atoms 3 and 4 also contained an excess of C^{13}, up to nearly one-half that in the other positions. Part of this labeled carbon undoubtedly was introduced by reentry into the cycle of isotopic oxalacetate generated in the cycle. Assuming recirculation in the cycle of such oxalacetate, one finds upon study of the cycle that at the end of the second circulation the C^{13} concentration in the carboxyl groups of this compound is one-third that of the non-carboxyl carbons, and that with further recirculation of the oxalacetate the ratio of the isotope concentration in the carboxyl carbons to that in the non-carboxyl carbons approaches one-half. The methyl carbon of acetate can thus contribute significantly to the 3 and 4 positions of the glycogen, without conversion to CO_2.

The condensation of doubly labeled acetate with oxalacetate would lead through the cycle to the formation of oxalacetate, all carbon atoms of which are labeled; decarboxylation of such oxalacetate yields pyruvate and hence glycogen likewise with all its carbons labeled. Again the experimental results are in agreement with this expectation. If the route described is the only one by which acetate carbon is being converted to glycogen, the isotope concentrations should be equal in each carbon atom of the glucose chain. The deviation of experimental observations from this anticipation, *i.e.* a greater abundance of isotope in carbon atoms 3 and 4 than in the other fractions of the glycogen, is reasonably explained by the introduction of extra C^{13} into these positions by CO_2 fixation. The fact that the observed C^{13} content of the respiratory CO_2 (Table IV) was high in the experiments with the doubly labeled acetate is consistent with the idea that the extra C^{13} came from CO_2. The high C^{13} content in the respiratory CO_2 resulted because both carbons of the acid were labeled and therefore the average excess C^{13} content was higher in this case than for the other acids administered.

In addition to comparing the agreement between the predictions of the scheme and the observed data relative to the distribution of the isotope in the carbon chain of the glucose unit, there is also opportunity for a similar comparison on the basis of recovery of the isotope in the respiratory CO_2.

The data on the respiratory CO_2 are shown in Table IV. The total respiratory CO_2 (column (*a*)) represents the sum of values for the three collection periods during which approximately equal quantities of CO_2 were obtained in each period. The total C^{13} shown in column (*b*) was obtained by summation of calculated C^{13} values for these three periods. In column (*c*) is shown the per cent of the administered C^{13} recovered in the respiratory CO_2. These values were calculated from column (*b*) and the mM of C^{13} administered, which may be calculated from the per cent C^{13} in the administered acid (Table II) and the amount of fatty acid given (Table I).

TABLE IV

Data on Respiratory CO_2

Fatty acid administered	Rat No.	C^{13} of respiratory CO_2 by collection periods			Total respiratory CO_2	Total C^{13} in respiratory CO_2	Administered C^{13} recovered in respiratory CO_2	Respiratory CO_2 from administered fatty acid
		1	2	3	(*a*)	(*b*)	(*c*)	(*d*)
		atom per cent excess	*atom per cent excess*	*atom per cent excess*	*mM*	*mM*	*per cent*	*per cent*
$CH_3C^{13}OOH$	33, 34	0.20	0.33	0.37	37.4	0.115	19 (24)*	12
"	38	0.20	0.44	0.39	11.2	0.047	16 (20)*	16
$C^{13}H_3COOH$	100, 101	0.16	0.35	0.32	37.1	0.100	35	14
"	102, 103	0.20	0.31	0.32	35.4	0.097	31	14
$C^{13}H_3C^{13}OOH$	51	0.34	0.60	0.59	18.3	0.094	20	12
"	63	0.41	0.66	0.61	20.8	0.119	24	13
"	64	0.42	0.64	0.61	22.2	0.127	26	13
"	69	0.41	0.69	0.74	18.6	0.114	22	13
"	70	0.70	0.90	0.62	19.5	0.137	27	15
"	71	0.54	0.95	0.91	19.4	0.158	34	18
"	72	0.50	0.76	0.72	20.1	0.134	29	14
"	73, 74	0.39	0.58	0.65	31.5	0.173	30	12
$CH_3CH_2CH_2C^{13}OOH$	53	0.17	0.27	0.19	23.1	0.050	20	22
"	67	0.20	0.36	0.33	20.0	0.061	27	31
$CH_3CH_2C^{13}H_2COOH$	81, 82	0.07	0.11	0.14	32.9	0.035	17	11
"	83, 84	0.06	(0.10)†	0.13	30.1	0.028	14	10
$CH_3C^{13}H_2CH_2COOH$	89, 90	0.26	0.31	0.26	31.1	0.086	29	26
"	91, 92	0.28	0.35	0.29	33.5	0.103	36	28

* The collection periods were 40 minutes each. The values in parentheses are the observed values multiplied by 5/4 to make the results comparable with the rest of the experiments.

† Assumed value.

The values in column (*c*) indicate the proportion of the total amount of the isotopic carbon of administered acetate which was oxidized to CO_2 in $2\frac{1}{2}$ hours under the conditions of the experiment. The values in column (*d*) present a different aspect in that they indicate what part of the respiratory CO_2 came from the fatty acid compared with that from other non-labeled materials in the metabolic pool of the animal. The figures in column (*d*) indicate simply the dilution of the administered isotope by nonisotopic carbon and were obtained by the following calculation.

$$\frac{\text{Atom \% excess C}^{13}\text{ in respiratory CO}_2}{\text{Atom \% excess C}^{13}\text{ in fed fatty acid}} \times 100$$

The values for the denominator are given in Table II, while those for the numerator may be calculated from Table IV, columns (*a*) and (*b*).

It should be emphasized that this calculation (column (*d*)) assumes (except for the doubly labeled acid, for which no such assumption is necessary since both carbon atoms contain an equal isotope concentration) that all carbons of the fatty acid chain in question are converted to CO_2 at equal rates.

As nearly as can be judged from the limited data available in Table IV, all types of labeled acetate are oxidized at about the same rate. The average for acetate is 27 per cent; *i.e.*, 27 per cent of the isotope was recovered in the respiratory CO_2 in $2\frac{1}{2}$ hours. Likewise that part of the respiratory CO_2 that came from the fatty acid was for all types of acetate the same, the average being 14 per cent of the total CO_2.

It is apparent that if the carboxyl carbon of acetate were actually converted to CO_2 more rapidly than the methyl carbon these values would have been higher for the carboxyl-labeled acid than for the methyl-labeled acid, and the values for the doubly labeled acetate would have been intermediate. The fact that all types of labeled acetate give the same results in the respiratory CO_2 is in agreement with the predictions of the cycle. Study of the schematic diagram will reveal that, at the end of the second circulation of oxalacetate through the cycle, one-half as much of the carboxyl carbon of the oxalacetate has been derived from the methyl carbon of the acetate as from the carboxyl carbon of the acetate. By further repeated circulation the total contribution of the carboxyl and methyl carbons of acetate to the carboxyl carbons of oxalacetate approaches equality. Since it is the carboxyl carbons of oxalacetate which yield CO_2 on passage through the cycle, the rate of conversion of the methyl carbon of acetate to CO_2 should approach that at which the carboxyl carbon is so converted.

From the foregoing it is concluded that both carbons of acetate are converted to CO_2 at approximately equal rates. This finding, as well as the results of the distribution of the isotope in the glucose, thus seems to be consistent with the suggestion that acetate may be oxidized by the tricarboxylic acid cycle.

It is noteworthy that the conversion of acetate to glycogen via the cycle cannot account for a net increase in glycogen from acetate carbon *per se*, since 2 moles of CO_2 are produced for each mole of acetate that enters the cycle. This is in agreement with the fact that glycogen is not deposited in the liver of the fasted rat following the feeding of acetate alone. As previously noted, however, the net change in glycogen in an animal may be as much dependent on the effect of the fed compound on the balance of the dynamic equilibrium of the body as it is on whether or not there is a mechanism for *net* transfer of carbons from the compound to the glycogen. The formation of glycogen in the traditional sense (*i.e.*, net glycogen deposition) contrasted with an actual transfer of carbon from a compound to glycogen as measured with isotopes may have no relation. When viewed in terms of actual movement of carbon to glycogen, it is apparent that short chain fatty acids and, in all probability, long chain fatty acids as well can be converted to carbohydrate. Bloch and Rittenberg (22) have shown that a long chain fatty acid (myristic acid) can give rise to acetyl groups in the intact organism. Furthermore it has been reported that acetate enters aspartic acid and glutamic acid, which are considered to be in biological equilibrium with carbohydrates by routes other than CO_2 fixation (23). These considerations together with present results with acetate indicate that glycogen carbon can be derived from long chain fatty acids.

It should be pointed out that the conversion of acetate to succinate by methyl to methyl condensation and a subsequent conversion of the succinate to pyruvate and then to glycogen would meet all the requirements of the present experimental data. Therefore on this basis there is no reason to exclude this mechanism. On the other hand the conversion of acetate to glycogen by a mechanism involving the formation of pyruvate by addition of a 1-carbon compound to the carboxyl of acetate (or a derivative) can be excluded. If this occurred, it would be expected that carboxyl-labeled acetate and probably carboxyl- and β-labeled butyrate would give rise to a α-labeled pyruvate. This type of pyruvate apparently was not the means of entrance of the acetate, for if it were, one would expect labeling to appear mainly in the 2,5 positions of the glucose. Actually this did not happen to a significant extent. On the other hand the results do not necessarily exclude the addition of a 1-carbon compound to the methyl carbon of acetate.

It is thus seen that, although the results are in agreement with the tricarboxylic acid cycle as a mechanism of conversion, this is by no means the only mechanism which would explain the results.

Pathways from Butyrate to Glycogen—There is a large body of experimental facts which indicates that fatty acids may be oxidized by β oxidation to acetate or derivatives of acetate (22, 24). If this occurs, it is reasonable to suppose that the resulting acetate would be converted to glycogen. With different types of labeled butyrate it should be possible by comparison with results obtained with the different kinds of labeled acetate to determine whether or not the β oxidation scheme suitably explains butyrate metabolism in intact animals. Thus carboxyl-labeled butyrate or β-labeled butyrate should be equivalent to carboxyl-labeled acetate and lead to the deposition of glycogen with isotope limited to the 3 and 4 carbon atoms of the glucose unit.

$$CH_3\cdot CH_2\cdot CH_2\cdot C^{13}OOH \rightarrow CH_3\cdot CO\cdot CH_2\cdot C^{13}OOH \rightarrow CH_3COOH + CH_3C^{13}OOH$$

$$CH_3\cdot C^{13}H_2\cdot CH_2\cdot COOH \rightarrow CH_3\cdot C^{13}O\cdot CH_2\cdot COOH \rightarrow CH_3C^{13}OOH + CH_3COOH$$

α-Labeled butyrate should be equivalent to methyl-labeled acetate and lead to the deposition of glycogen with isotope preponderantly in carbon atoms 1, 2, 5, and 6.

$$CH_3\cdot CH_2\cdot C^{13}H_2\cdot COOH \rightarrow CH_3\cdot CO\cdot C^{13}H_2\cdot COOH \rightarrow CH_3COOH + C^{13}H_3COOH$$

The results shown in Table II are in complete agreement with these proposals and offer support to the idea that butyrate undergoes β oxidation and that the resulting 2-carbon compound may be converted to glycogen via the tricarboxylic acid cycle through reactions similar to those previously described for acetate. It is realized, of course, that what are observed in these tests are the end-results and that no real information is given on intermediate steps. There may be other mechanisms which would fulfil the requirements of the data, but at present the β oxidation theory appears adequate.

The present results apparently rule out, at least for the conditions of these experiments, the formation of glycogen by ω oxidation of butyrate to succinate as suggested by Blixenkrone-Möller (25). Whereas the results with carboxyl- and α-labeled butyrate could be explained by this mechanism, it cannot explain the results with the β-labeled butyrate. By ω oxidation to succinate, isotope from β-labeled butyrate would be located predominantly in positions 1, 2, 5, and 6 of the glycogen, as illustrated by the following equations.

$$CH_3\cdot C^{13}H_2\cdot CH_2\cdot COOH \longrightarrow COOH\cdot C^{13}H_2\cdot CH_2\cdot COOH \longrightarrow$$

$$\begin{array}{ccccc} COOH\cdot C^{13}H_2\cdot CO\cdot COOH & & C^{13}H_3\cdot CO\cdot COOH & & \\ \text{or} & \longrightarrow & \text{or} & \longrightarrow & C^{13}\cdot C^{13}\cdot C\cdot C\cdot C^{13}\cdot C^{13} \\ COOH\cdot C^{13}O\cdot CH_2\cdot COOH & & CH_3\cdot C^{13}O\cdot COOH & & \text{Glucose} \end{array}$$

Actually, as already noted, a significant excess of C^{13} could be detected only in positions 3 and 4. It should be noted that this distribution probably would not result by ω oxidation even if the resulting dicarboxylic acid were not symmetrical. From an unsymmetrical C_4-dicarboxylic acid either of the two types of pyruvate as illustrated above would be expected and neither would give a glucose unit labelled in positions 3 and 4. Furthermore the oxidation of butyrate to succinate, followed by cleavage in the center, to 2 molecules of acetate is not likely, because this mechanism would be expected to give a glucose from β-labeled butyrate like that obtained from methyl-labeled acetate, whereas it did not.

Metabolism of butyrate via acetate would not directly provide for a net increase in carbohydrate precursors, but such an increase if it does occur (as indicated, for example, by the work of Blixenkrone-Möller, wherein glucogenesis was reported in perfused livers following the administration of butyrate) could be an indirect effect of the fatty acid. The presence of butyrate may exert a sparing effect and cause the accumulation of glycogen from available carbohydrate or carbohydrate precursors derived from protein.

If butyrate is metabolized via acetate, one might expect, from the results on the three types of labeled acetate, that all carbon atoms of butyrate would be converted to CO_2 at approximately equal rates. From the data on respiratory CO_2 (Table IV) it would appear that the carboxyl and β-carbons of butyrate are burned much more rapidly than is the α-carbon. Unfortunately, the experiments with the α-labeled butyrate are not entirely comparable to those in which the carboxyl- and β-labeled acids were fed, since considerably smaller amounts of the α-labeled butyrate were administered and also this fatty acid was contaminated with appreciable amounts of non-isotopic propionate (see Table I). Both these factors would lower the relative amount of labeled carbon in relation to the total metabolic pool and thus the C^{13} in the respiratory CO_2 would be less concentrated. As a consequence of this, as judged by column (*d*), Table IV, which gives the dilution in the respiratory CO_2 of administered isotope by non-isotopic metabolic CO_2, the oxidation of the α-carbon would be indicated to be relatively lower than for the other positions. Actually, however, a proportionally greater oxidation of non-isotopic material may have been the real cause of this difference. The values for the per cent of the administered isotope recovered in the CO_2 (column (*c*)) also indicate a slower oxidation of the α-carbon, but no definite conclusion seems justified on the basis of the two results, for it is seen in column (*c*) that there is a great deal of variation in the results from one experiment to another. This variation may in part be caused by variation in absorption. Thus at present there is no reliable indication that the rate of oxidation differs for the individual positions of the butyrate molecule and this question must be left open.

Considered as a whole, these studies with acetate and butyrate which have been performed on intact normal animals have yielded results which agree remarkably well with predictions from schemes which have been derived for the most part from studies *in vitro*. The results give some evidence that the tricarboxylic acid cycle is an active mechanism in the normal animal; also the evidence lends support to the conventional schemes of glycolysis. It should be borne in mind that, although these studies were made on whole, fasted animals, they probably represent in a large part the metabolism of the liver. It is conceivable that studies of longer duration with glycogen from other tissues might yield results which differed from the present results. Conceivably kidney, heart, etc., may follow metabolic pathways with some variations from the metabolism of liver.

SUMMARY

The glycogen of rat liver was isolated following feeding, by stomach tube, of glucose plus either acetate or butyrate labeled with C^{13}. The glycogen was hydrolyzed to glucose, and the latter degraded.

After the administration of $CH_3 \cdot C^{13}OOH$ or $CH_3 \cdot CH_2 \cdot CH_2 \cdot C^{13}OOH$ as well as after the administration of $CH_3 \cdot C^{13}H_2 \cdot CH_2 \cdot COOH$, all of the excess isotopic carbon in the glycogen was found in positions 3 and 4 of the glucose unit. Since these are the same positions as for CO_2 fixation, and since considerable amounts of isotopic carbon appeared in the respiratory CO_2, it was impossible from location of C^{13} in the glycogen to determine whether there was an additional pathway of conversion of fatty acid carbon to glycogen.

With $C^{13}H_3 \cdot COOH$ or $C^{13}H_3 \cdot C^{13}OOH$ or $CH_3 \cdot CH_2 \cdot C^{13}H_2 \cdot COOH$, all degradation fractions of the glycogen contained excess C^{13}. This is considered direct evidence that at least the α-carbon atoms of acetate and butyrate can enter glycogen by a pathway other than CO_2 fixation.

The distribution of isotope found in liver glycogen is consistent with formation of 3-carbon carbohydrate fragments from acetate and butyrate via the tricarboxylic acid cycle, and synthesis of the 6-carbon glycogen units from two 3-carbon fragments through reversal of the conventional reactions of glycolysis.

The results after the administration of butyrate are consistent with the β oxidation of butyrate to 2 molecules of acetate, but inconsistent with ω oxidation of butyrate to succinate.

These observations have been made with intact animals and provide some evidence for the functioning, under *in vivo* conditions, of schemes based largely on *in vitro* studies.

We wish to thank Dr. Walton Shreeve, Miss Carol Carlson, Miss Eleanor Davies, Miss Bernice Theissen, and Miss Margaret Cook for help with a number of the chemical determinations, and Dr. A. O. C. Nier of the Department of Physics, University of Minnesota, for most of the mass spectrometer measurements.

BIBLIOGRAPHY

1. Buchanan, J. M., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, **150**, 413 (1943).
2. Wood, H. G., Lifson, N., and Lorber, V., *J. Biol. Chem.*, **159**, 475 (1945).
3. Lifson, N., Lorber, V., and Wood, H. G., *Federation Proc.*, **4**, 47 (1945).
4. Lorber, V., Lifson, N., and Wood, H. G., *J. Biol. Chem.*, **161**, 411 (1945).
5. Lifson, N., Lorber, V., Sakami, W., and Wood, H. G., *Federation Proc.*, **6**, 152 (1947).
6. Osborn, O. L., Wood, H. G., and Werkman, C. H., *Ind. and Eng. Chem., Anal. Ed.*, **5**, 247 (1933).
7. Anker, H. S., *J. Biol. Chem.*, **166**, 219 (1946).
8. Cramer, R. D., and Kistiakowsky, G. B., *J. Biol. Chem.*, **137**, 549 (1941).
9. Stahly, G. L., Osborn, O. L., and Werkman, C. H., *Analyst*, **59**, 319 (1934).
10. Sakami, W., Evans, W. E., and Gurin, S., *J. Am. Chem. Soc.*, **69**, 1110 (1947).
11. Adkins, H., and Folkers, K., *J. Am. Chem. Soc.*, **53**, 1095 (1931).
12. Folkers, K., and Adkins, H. J., *J. Am. Chem. Soc.*, **54**, 1145 (1932).
13. Norris, J. F., *Am. Chem. J.*, **38**, 627 (1907).
14. Bischoff, C. A., *Ber. chem. Ges.*, **28**, 2622 (1895).
15. Stetten, D., Jr., and Boxer, G. E., *J. Biol. Chem.*, **155**, 231 (1944).
16. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, **100**, 485 (1933).
17. Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, **136**, 509 (1940).
18. Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, **45**, 365 (1921).
19. Folin, O., and Malmros, H., *J. Biol. Chem.*, **83**, 115 (1929).
20. Osborn, O. L., and Werkman, C. H., *Ind. and Eng. Chem., Anal. Ed.*, **4**, 421 (1932).
21. Ball, E. G., Tucker, H. F., Solomon, A. K., and Vennesland, B., *J. Biol. Chem.*, **140**, 119 (1941).
22. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **155**, 243 (1944).
23. Rittenberg, D., and Bloch, K., *J. Biol. Chem.*, **157**, 749 (1945).
24. Medes, G., Weinhouse, S., and Floyd, N. F., *J. Biol. Chem.*, **157**, 35 (1945).
25. Blixenkrone-Möller, N., *Z. physiol. Chem.*, **252**, 137 (1938).

INFLUENCE OF TESTOSTERONE PROPIONATE ON THE PLASMA AND LIVER PROTEINS OF HYPOTHYROID RATS*

By JAMES H. LEATHEM

(*From the Bureau of Biological Research, Rutgers University, New Brunswick*)

(Received for publication, July 23, 1948)

Clinically a relationship between thyroid activity and blood protein levels has been indicated by a hyperproteinemia in myxedema (1) and is correlated with an increase in globulin levels (2). This relationship has been observed in dogs (3) and has been seen repeatedly in thyroidectomized rats and in rats fed antithyroid drugs (4–6). Since a decrease in weight of the seminal vesicles followed thyroidectomy (7) and thiourea feeding (8), the implication that androgen production is waning in hypothyroidism invited the study of testosterone propionate action in rats fed antithyroid drugs. These data aid in determining whether hypothyroidism or hypogonadism is of primary importance in causing the rise in plasma globulin associated with both conditions. Furthermore, an effect might be anticipated, since androgen administration induced nitrogen retention in a cretin (9).

Liver size and the ratio of liver protein to body weight increased after thiouracil feeding (10). In view of the known protein anabolic action of androgens (11) studies concerning liver protein in the hypothyroid state with and without concomitant androgen are presented.

EXPERIMENTAL

Male rats of the Long-Evans strain were used when 150 days old and were kept in metabolism cages for measurement of daily food intake. The rats were raised on Purina fox chow (20.1 to 23.9 per cent protein) and it was also fed during the experimental period. Thiourea and thiouracil,[1] were added as 0.5 per cent of the diet, and the rats fed *ad libitum*. Other groups received thiourea or thiouracil and testosterone propionate[1] or were normal rats with and without androgen, but all were pair-fed with the group on the goitrogic substance alone, as thiourea and thiouracil reduce

---

* Supported by the Protein Metabolism Fund of the Bureau of Biological Research, Rutgers University, initially, and completed under a contract with the Office of Naval Research, Navy Department.

[1] Thiouracil (deravet) was supplied by Dr. Mark Welsh, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, and testosterone propionate (perandren, Ciba) was supplied by Dr. E. Oppenheimer, Ciba Pharmaceutical Products, Summit, New Jersey.

food intake. After a 20 to 27 day experimental period the rats were lightly anesthetized with ether and bled from the heart. Hematocrit, non-protein nitrogen, total plasma protein, albumin, and globulin were determined. Albumin and globulin were separated by the Howe method (12), as modified by Robinson, Price, and Hogden (13). Nitrogen values were corrected for non-protein nitrogen and converted to protein by use of the factor 6.25. Liver nitrogen was determined after the organ had been dried to constant weight at 95° and ground to uniform consistency.

Results

Thiourea feeding reduced food intake to an average of 273 gm. per rat for 20 days, as compared to the 335 gm. consumed by rats eating *ad libitum*. The reduced food intake resulted in a loss in body weight which, however, was greater in the thiourea-fed rats than in pair-fed controls. Testosterone propionate administered subcutaneously at a level of 0.1 mg. daily did not prevent weight loss, although this androgen caused retention of urinary nitrogen in men on a restricted caloric intake (14).

Thiourea-fed rats exhibited a decrease in hematocrit and an increase in non-protein nitrogen, but an increase was not observed in normal rats on reduced food intake. Of the deviations from normal in rats fed thiourea, only non-protein nitrogen remained within the normal range in rats receiving both thiourea and androgen. Total plasma protein was increased by thiourea, owing to an increase in concentration of plasma globulin, while plasma albumin levels were unaltered (Table I). The androgen had a tendency to reduce the elevated plasma protein levels, but the differences were not significant. In the normal rat on restricted food intake, the plasma globulin level had a tendency to be higher than in rats fed *ad libitum*, being 2.72 gm. per cent compared with 2.51 gm. per cent. Androgen administration reduced the plasma globulin to a normal concentration, but in all cases the data are of border line significance.

Although the daily dosage of 0.1 mg. of testosterone propionate was adequate in maintaining weight of the seminal vesicles in castrated rats, this dosage was essentially ineffective in maintaining normal plasma protein levels in hypothyroid rats. It seemed advisable to repeat the entire experiment with 0.5 mg. of androgen daily, but the results generally duplicated the data obtained with the lower dosage.

The possibility that a toxic action of thiourea may have masked the action of testosterone on the concentration of plasma protein prompted the use of thiouracil. Thiouracil fed at 0.5 per cent reduced food intake to 300 gm. per rat over 20 days, causing a modest loss in body weight, which was duplicated by pair-fed controls. 0.5 mg. of testosterone propionate was used alone and in combination with thiouracil. Loss in body weight was not prevented by testosterone propionate, but the increase in non-protein nitrogen and the decrease in hematocrit induced by thiouracil could be largely prevented by androgen administration. Thiouracil increased plasma globulin concentrations in the absence of a change in plasma albumin levels (Table II), and the administration of androgen was without effect. The increase in plasma globulin in rats due to thiouracil is associated with an increase in α-globulin (6), and α-naphthylthiourea

TABLE I

Plasma Protein Concentrations in Rats Treated with Thiourea (0.5 Per Cent) and Testosterone Propionate (0.1 Mg.)

No. of rats	Treatment	Body weight*	Hematocrit	Non-protein nitrogen	Total protein	Albumin	Globulin
		gm.	*per cent*	*mg. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*
12	Thiourea	294–256	40.5	68	6.66	3.49	3.17
			±1.0†	±3.4	±0.10	±0.12	±0.09
12	" + testosterone propionate	294–259	41.3	60	6.41	3.38	3.02
			±1.1	±3.2	±0.11	±0.15	±0.09
12	Normal, pair-fed	288–271	45.4	58	6.10	3.37	2.72
			±0.8	±3.2	±0.07	±0.06	±0.09
6	" + testosterone propionate	303–280	46.3	57	5.93	3.36	2.57
			±1.2	±4.0	±0.07	±0.14	±0.14

* Initial and final.

† $\epsilon = \sqrt{\Sigma d^2/(n(n-1))}$.

TABLE II

Plasma Protein Concentrations in Rats Treated with Thiouracil (0.5 per cent) and Testosterone Propionate (0.5 Mg.)

Each group consisted of twelve rats.

Treatment	Body weight*	Hematocrit	Non-protein nitrogen	Total protein	Albumin	Globulin
	gm.	*per cent*	*mg. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*
Thiouracil	336–326	44.4	66	6.81	3.26	3.55
		±0.5	±1.7	±0.09	±0.08	±0.17
" + testosterone propionate	331–317	46.4	58	6.95	3.36	3.59
		±0.6	±2.5	±0.10	±0.07	±0.13
Normal, pair-fed	336–315	48.7	56	6.04	3.19	2.85
		±0.6	±1.1	±0.11	±0.08	±0.14
" + testosterone propionate	324–312	49.7	54	6.08	3.23	2.85
		±0.7	±2.7	±0.08	±0.10	±0.09

* Initial and final.

incites a similar reaction in dogs (15). The antithyroid drugs have varied toxicities, and thus their action might be to stimulate the adrenal, the secretions of which increase serum globulin (16). The action of antithyroid drugs on plasma protein levels would seem to be independent of adrenal excitation, however, since these compounds reduce adrenal activity (17, 18). Furthermore, normal globulin levels can be maintained in hypophysectomized rats with thyroxine (4), and adrenocorticotropic hormone does not release globulin in these animals (19).

Table III reveals that thiouracil will increase liver weight significantly without changing the percentage of water or protein. Consequently the total liver protein in the body and the liver protein in gm. per 100 gm. of body weight were significantly above normal. Testosterone propionate was essentially without effect on the liver water or protein in thiouracil-fed or normal pair-fed rats. The increase in liver weight induced by thiouracil was less pronounced when androgen was administered concomitantly. The increase in the ratio of liver protein to body weight is obtained only after thiouracil feedings (10), thyroidectomy favoring liver atrophy (20). The thiouracil action is surprising in the wake of reduced food intake, since the liver can be depleted of protein quickly (21). The increase in liver weight might also be aided by an increase in fat (22) and glycogen (23), but contradictory data have also been reported regarding these components in the liver (23, 18).

TABLE III

Influence of Thiouracil (0.5 Per Cent) and Testosterone Propionate (0.5 Mg.) on Liver of Adult Rats

Each group consisted of seven rats.

Treatment	Liver weight		Water	Total protein	Liver protein	
	gm.	*gm. per 100 gm. body weight*	*per cent*	*gm.*	*per cent dry weight*	*gm. per 100 gm. body weight*
Thiouracil	15.2	4.4	71.0	2.93	66.9	0.887 (0.801–0.999)
	±0.9	±0.3	±0.3	±0.13	±1.2	
" + testosterone propionate	13.2	4.2	70.3	2.63	67.2	0.835 (0.714–0.910)
	±0.7	±0.3	±0.2	±0.11	±1.2	
Normal, pair-fed	10.9	3.3	70.1	2.27	69.9	0.719 (0.652–0.788)
	±0.6	±0.2	±0.6	±0.10	±1.7	
" + testosterone propionate	10.9	3.5	69.9	2.27	70.1	0.717 (0.626–0.856)
	±0.9	±0.3	±0.3	±0.13	±1.5	

SUMMARY

Hypothyroidism induced by feeding thiourea or thiouracil resulted in an increased concentration of total plasma protein, plasma globulin, and

non-protein nitrogen. Plasma albumin concentration was not changed, but the hematocrit decreased. An increase in liver size, while water and protein remained at normal percentages, resulted in an increased liver protein in the body after thiouracil feeding. Testosterone propionate in doses of 0.1 and 0.5 mg. daily did not alter the plasma or liver proteins of hypothyroid rats, except to reduce the non-protein nitrogen. The slight rise in plasma globulin sometimes associated with restricted food intake was prevented by testosterone propionate administration, and the increase in liver weight induced by thiouracil was less pronounced when androgen was administered concomitantly.

BIBLIOGRAPHY

1. Podhradsky, L., *Acta med. Scand.*, **104**, 137 (1940).
2. Lewis, L. A., and McCullagh, E. P., *Am. J. Med. Sc.*, **208**, 727 (1944).
3. Goldberg, I., *Compt. rend. Soc. biol.*, **128**, 1135 (1938).
4. Levin, L., and Leathem, J. H., *Am. J. Physiol.*, **136**, 306 (1942).
5. Leathem, J. H., *Endocrinology*, **36**, 98 (1945).
6. Moore, D. H., Levin, L., and Smelser, G. K., *J. Biol. Chem.*, **157**, 723 (1945).
7. Smelser, G. K., *Anat. Rec.*, **74**, 7 (1939).
8. Leathem, J. H., *Proc. Soc. Exp. Biol. and Med.*, **61**, 203 (1946).
9. Howard, J. E., Wilkins, L., and Fleischmann, W., *Tr. Assn. Am. Physn.*, **57**, 212 (1942).
10. Leathem, J. H., and Seeley, R. D., *Endocrinology*, **42**, 150 (1948).
11. Kochakian, C. D., in Harris, R. S., and Thimann, K. V., Vitamins and hormones, New York, **4**, 256 (1946).
12. Howe, P. E., *J. Biol. Chem.*, **49**, 109 (1921).
13. Robinson, H. W., Price, J. W., and Hogden, C. G., *J. Biol. Chem.*, **120**, 481 (1937).
14. Butler, A. M., Talbot, N. B., MacLachlan, E. A., Appleton, J. E., and Linton, M. A., *J. Clin. Endocrinol.*, **5**, 327 (1945).
15. Chanutin, A., Gjessing, E. C., and Ludewig, S., *Proc. Soc. Exp. Biol. and Med.*, **64**, 172 (1947).
16. White, A., and Dougherty, T. F., *Endocrinology*, **36**, 207 (1945).
17. Deane, H. W., and Greep, R. O., *Endocrinology*, **41**, 243 (1947).
18. Glock, G. E., *Nature*, **156**, 508 (1945).
19. Li, C. H., and Reinhardt, W. O., *J. Biol. Chem.*, **167**, 487 (1947).
20. Leblond, C. P., and Hoff, H. E., *Endocrinology*, **35**, 229 (1944).
21. Kosterlitz, H. W., and Cramb, I. D., *J. Physiol.*, **102**, 18 (1943).
22. Handler, P., *J. Biol. Chem.*, **173**, 295 (1948).
23. May, L. G., Moseley, R. W., and Forbes, J. C., *Endocrinology*, **38**, 147 (1946).

STUDIES ON THE STRUCTURE OF LYCOMARASMIN

By D. W. WOOLLEY

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

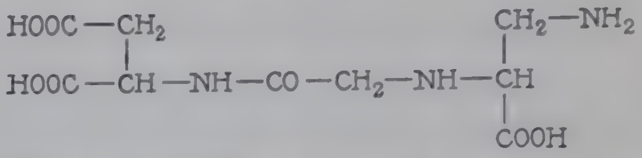
(Received for publication, July 3, 1948)

Plattner and Clauson-Kaas (1) have isolated a substance called lycomarasmin from the culture filtrates of the phytopathogenic fungus *Fusarium lycopersici*. When this organism infects tomato plants, it causes the leaves to wilt and curl, and this is believed to be due to the formation of a toxin which can be demonstrated in the intercellular spaces of the host (2). A similar toxin is formed by the fungus growing in synthetic medium, because filtrates of such cultures cause excised tomato leaves to wilt and curl just as they do on infected plants. The lycomarasmin which was isolated from these filtrates had the same powers against tomato leaves and represented a considerable portion of the toxic activity of the culture.

Plattner and Clauson-Kaas (3) found that lycomarasmin was a small molecule of empirical formula $C_9H_{16}O_7N_3$, and that it yielded on hydrolysis two amino acids, glycine and aspartic acid. Since these constituents would leave only 3 carbon atoms and 1 nitrogen atom unaccounted for, and since mild hydrolysis led to the formation of 1 molecule of ammonia, a labile grouping was indicated in the molecule, and indeed, the failure of the toxin to give a blue color with ninhydrin, or N_2 in the Van Slyke reaction with HNO_2, suggested that the unidentified 3 carbon and 1 nitrogen did not belong to a conventional amino acid such as alanine. The instability of lycomarasmin was well illustrated by the finding that heating it in water, or merely allowing the free acid to stand for long periods in aqueous solution, led to the formation of a biologically inactive compound, $C_9H_{12}O_7N_2$, which differed from the toxin by the elements of NH_3. This latter compound was designated Substance I. Largely on the basis of these findings, a structure was proposed for lycomarasmin (Fig. 1).

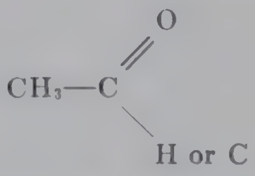
Since strepogenin had been shown to be quite probably a derivative of glutamic acid (4), the hypothesis occurred to us that lycomarasmin and strepogenin might be structural analogues in which the glutamic acid of the growth factor strepogenin was replaced by aspartic acid in the inhibitory or toxic lycomarasmin. The occurrence of glycine in both substances (3, 5) was interesting in this connection. Therefore, an investigation of the structure of lycomarasmin was begun in this laboratory in the hope of throwing light on the constitution of strepogenin. Lycomarasmin had been obtained in pure form, whereas strepogenin had not.

The results of these studies do not seem to be compatible with the structure proposed by Plattner and Clauson-Kaas (3), which was admittedly tentative. In the first place, lycomarasmin can be shown to be a derivative of asparagine. If the toxin is treated with a solution of KOBr, a Hofmann degradation apparently occurs, and aspartic acid can no longer be found in the hydrolytic products. Substance I of Plattner and Clauson-Kaas, on the other hand, maintains its aspartic acid after treatment with hypobromite. The location of the 3rd N atom thus is indicated. In the second place, lycomarasmin yields iodoform, whereas Substance I does not. Therefore, the grouping



$$\mathrm{HOOC{-}\overset{\overset{\displaystyle HOOC{-}CH_2}{|}}{CH}{-}NH{-}CO{-}CH_2{-}NH{-}\underset{\underset{\displaystyle COOH}{|}}{\overset{\overset{\displaystyle CH_2{-}NH_2}{|}}{CH}}}$$

FIG. 1. Structure of lycomarasmin according to Plattner and Clauson-Kaas

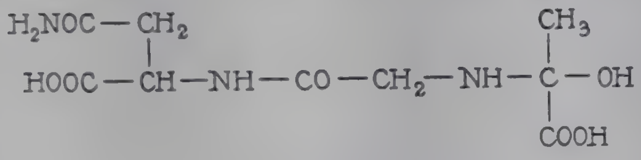


must be present in (or derivable from) the former but absent from the latter. As Plattner and Clauson-Kaas (1) have indicated, no free aliphatic NH_2 group seems to be present, so that when either lycomarasmin or Substance I is treated with HNO_2, neither glycine nor aspartic acid is destroyed. When lycomarasmin is tosylated with *p*-toluenesulfonyl chloride in warm, alkaline solution, a simultaneous cleavage takes place, and an ethyl acetate-soluble tosyl derivative is formed. On careful hydrolysis this derivative yields tosylglycine and aspartic acid, from which it may be concluded that the tosyl derivative is probably tosylglycylaspartic acid. The unstable 3 C moiety of the toxin is probably lost during the acylation. Substance I apparently contains a double bond, because it adds bromine in the cold to give a halogenated derivative.

In view of these facts, the structures of Fig. 2 are proposed to represent lycomarasmin and Substance I. The toxin thus would contain a derivative of the unstable amino acid, α-hydroxyalanine. This is stabilized in such a way as not to lose ammonia by the replacement of 1 hydrogen atom of its amino group by the nitrogen-free part of a glycine residue. This new and unstable amino acid has been suspected to occur in another product of fungi, namely ergotamine, as demonstrated by Jacobs and Craig (6).

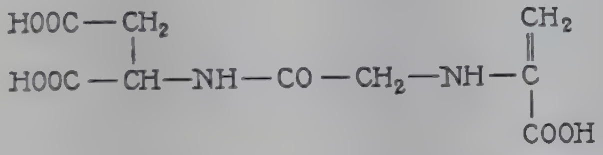
A second motive which stimulated the work was the realization that lycomarasmin was a small molecule, composed of amino acid residues, with marked biological activity. It might thus serve well as a model for the study of some aspects of the structure and biological activities of proteins. Being a small molecule, it could be subjected to searching proof of structure and to chemical rearrangement of the component parts by totally synthetic means much more readily than is true for the proteins.

The easy conversion of lycomarasmin into Substance I merely by heating or standing is an interesting analogy to heat-inactivation of more complex peptides or proteins. Thus the active toxin is converted to an inactive product in the reaction. If the formulation in Fig. 2 is correct, the chemical change in this case would involve the loss of water between the unstable tertiary alcoholic group and the H of the adjoining methyl group. The ammonia which is liberated during the reaction would arise by concomitant hydrolysis of the amide group of the asparagine residue.



$$\mathrm{HOOC{-}\overset{\displaystyle H_2NOC{-}CH_2}{\overset{|}{C}H}{-}NH{-}CO{-}CH_2{-}NH{-}\underset{\underset{\displaystyle COOH}{|}}{\overset{\overset{\displaystyle CH_3}{|}}{C}}{-}OH}$$

Lycomarasmin



$$\mathrm{HOOC{-}\overset{\displaystyle HOOC{-}CH_2}{\overset{|}{C}H}{-}NH{-}CO{-}CH_2{-}NH{-}\underset{\underset{\displaystyle COOH}{|}}{\overset{\overset{\displaystyle CH_2}{\|}}{C}}}$$

Substance I

FIG. 2. Proposed structures for lycomarasmin and for Substance I of Plattner and Clauson-Kaas.

An attempt was made to synthesize lycomarasmin as represented in Fig. 2 by the condensation of ethyl-α-acetoxy-α-bromopropionate with the methyl ester of glycylasparagine, followed by selective saponification of the ester groups. A substance was isolated from the reaction mixture which had approximately the theoretical amount of nitrogen for lycomarasmin. Furthermore, its biological activity in causing wilting and curling of tomato leaves was equal to that of lycomarasmin. However, too much weight cannot be given this latter finding, because, as will be shown in a subsequent paper, several substances closely related to lycomarasmin possess biological activity. The difficulty in establishing beyond question the identity of the synthetic product with lycomarasmin was that the proposed structure contained 2 asymmetric carbon atoms, and although optically active asparagine was used in the synthesis, the bromoacetoxypropionate was racemic. The rather unstable nature of this compound prevented adequate purification or resolution. Therefore, the synthetic product must have been a mixture of diastereomers, and this probably explains the observed fact that the synthetic substance was more soluble in water than was the natural product.

EXPERIMENTAL

Source of Material—Crystalline lycomarasmin and Substance I were isolated according to the directions of Plattner and Clauson-Kaas (1) from 3 month-old culture filtrates of *Fusarium lycopersici*. A transplant of this organism was very kindly supplied by Dr. G. W. Irving, Jr., of the United States Department of Agriculture. The substances agreed well in all physical properties with those described by Plattner and Clauson-Kaas.

Amino Acid Composition of Lycomarasmin and Substance I—In order to ascertain whether glycine and aspartic acid were the only amino acids liberated on acid hydrolysis of lycomarasmin and Substance I, 5 mg. samples were digested in sealed tubes with 22 per cent HCl at 115° for 20 hours. The hydrolysates were concentrated to dryness under reduced pressure, dissolved in water, again concentrated, and finally dissolved, neutralized, and adjusted to 5 cc. These preparations were then partitioned on paper strips according to the directions of Consden, Gordon, and Martin (7). With either butanol or with phenol as the organic phase, only two colored zones, corresponding to the positions of glycine and aspartic acid, were found. Quantitative estimation of the amounts of these two amino acids by microbiological methods was not satisfactory because of the great difficulty experienced in obtaining complete hydrolysis. As the rigor of the treatment was increased, the yield of amino acids went up, so that with the conditions outlined in the paragraph above, almost twice as much of both amino acids were found as with hydrolysates prepared by refluxing with 20 per cent HCl overnight. Even with the more vigorous hydrolysis, Substance I yielded values of 30 per cent for aspartic acid and 17 per cent for glycine. Theory, 51 and 29. The aspartic acid was estimated by the method of Hac and Snell (8). At the time these experiments were being performed, no microbiological method for the determination of glycine had been published, but a satisfactory one was devised by use of the same organism and basal medium as were employed for the aspartic acid determination. Glycine was omitted and aspartic acid was added to the stock solutions. In addition, 0.1 mg. of an acid hydrolysate of casein was added per tube. The use of the organism was suggested by Dr. M. S. Dunn, who has since published a similar method for the microbiological assay of glycine (9). Despite the difficulty of obtaining complete liberation of amino acids from the peptides lycomarasmin and Substance I, the molar ratio of aspartic acid to glycine in the hydrolysates was 1:1.

Hofmann Degradation of Lycomarasmin and of Substance I to Demonstrate Presence of Substituted Asparagine in Former—2.5 mg. samples of lycomarasmin and of Substance I were dissolved in 2.5 cc. of water at pH 7, and the solutions were cooled to 0° and mixed with a cold solution of 100 mg. of bromine in 2 cc. of N KOH. The reaction was allowed to proceed for half an hour at 0°, and then for an hour at 40°. A slight excess of HCl was added, and the solutions were evaporated to dryness under reduced pressure. The residues were taken up in 22 per cent HCl, hydrolyzed, and assayed for aspartic acid. At the same time, controls were run in which the KOH but not the bromine was used.

Under these conditions, no aspartic acid was found in the reaction product from lycomarasmin, while that from Substance I retained about 75 per cent of this amino acid. When asparagine and aspartic acid were treated with KOBr in the same fashion, all of the former and about a third of the latter were destroyed.

Iodoform from Lycomarasmin—25 mg. of lycomarasmin were dissolved in 2 cc. of 2 N NaOH, and the solution was treated with iodine dissolved in 15 per cent aqueous KI until a yellow color persisted. The mixture was then warmed to 60° for a few minutes. During the warming, a yellow precipitate formed, and the odor of iodoform was plainly discernible. The precipitate was found to consist of yellow, hexagonal plates which melted at 119°.

When 100 mg. of Substance I were tested in the same fashion, no evidence of iodoform was obtained.

Failure to Destroy Glycine or Aspartic Acid by Treatment of Lycomarasmin with HNO_2—20 mg. of lycomarasmin dissolved in 1 cc. of water plus 3 cc. of glacial acetic acid were treated with 35 mg. of $NaNO_2$ dissolved in 2 cc. of water. After a reaction time of 0.5 hours had elapsed, excess reagents were destroyed by evaporation under reduced pressure and by addition of 40 mg. of alanine. Hydrolysis and estimation of glycine and aspartic acid were carried out as indicated earlier, and 4.8 mg. of the former and 7.2 mg. of the latter were found. A control in which the $NaNO_2$ was omitted was found to yield the same values. Therefore, the amino groups of glycine and of aspartic acid were not free in lycomarasmin.

Control experiments showed that under these conditions, both free glycine and free aspartic acid were so altered by HNO_2 as not to be determinable in the assays. Furthermore, it was observed that HCl could not be used successfully in place of acetic acid in the complete deamination of these substances.

Isolation of Tosyl Glycine by Tosylation and Subsequent Partial Hydrolysis of Lycomarasmin—200 mg. of lycomarasmin were dissolved in water at pH 7, and 12 cc. of 1 N NaOH were added and the mixture heated to 70°. 1 gm. of *p*-toluenesulfonyl chloride was added, and the suspension was shaken until the acid chloride had disappeared (about 5 minutes). The cooled, acidified reaction mixture was extracted six times with ethyl acetate, and the extract was freed of solvents under reduced pressure. The material so obtained was a water-soluble substance which could not be crystallized. It was, therefore, dissolved in 13 cc. of 0.5 N HCl and 13 cc. of formic acid, and the solution was refluxed for 48 hours. The solvents were removed under reduced pressure, and the residue taken up in 5 cc. of water. A crystalline precipitate was removed and the aqueous phase was extracted four times with ethyl acetate. The extracted material was combined with the crystals and the whole recrystallized from water to yield 73 mg. melting at 142°, the same as did tosylglycine.

$C_9H_{11}O_4NS$. Calculated, C 47.2, H 4.8, N 6.1; found, C 47.2, H 4.9, N 6.1

The aqueous phase from the hydrolysate was assayed for aspartic acid and found to contain 51 mg. of it. It seemed probable that a 3 carbon moiety was cleaved under the conditions of the acylation, and that the oily product subsequently extracted by ethyl acetate was largely tosylglycylaspartic acid.

Synthesis of Substance with Biological Activity and Several Other Properties of Lycomarasmin. (*a*) *Ethyl-α-acetoxy-α-bromopropionate*—6.6 gm. of acetyllactic acid and 2 gm. of red phosphorus were treated dropwise, with stirring and cooling, with 16 gm. of bromine. After the rather vigorous reaction, with evolution of HBr, had subsided, the mixture was allowed to stand overnight. Excess bromine and HBr were removed under reduced pressure, and 100 cc. of cold water were added. The free acid was extracted with ether, and the solvent was removed from the extract under reduced pressure. The yellow, oily product (7.7 gm.) could not be purified further because of its tendency to decompose. It showed a neutral equivalent of 191, whereas theory for α-acetoxy-α-bromopropionic acid was 211. In order to determine whether any of the desired substance was present, 240 mg. were dissolved in 5 cc. of 1 N NaOH and held at 90° for 1 hour. Acidification, extraction of the acids, and preparation of the *p*-nitrophenylhydrazone gave the characteristic derivative of pyruvic acid which melted at 210–213°. Hydrolysis of the acetyl group of α-acetoxy-α-bromopropionic acid should lead to an unstable intermediate which, by loss of HBr, should yield pyruvic acid.

The ethyl ester was prepared by allowing the acid to stand in a large excess of absolute alcohol for 3 months at 4°, but a better procedure was to add 20 cc. of absolute alcohol to the reaction mixture directly after the bromination before any moisture was admitted. The crude ester was then collected in ether, washed with water, dried, and freed of solvents and volatile impurities *in vacuo*. The product was a neutral yellow oil which slowly liberated acid on exposure to water.

(*b*) *Condensation with Methyl Ester of Glycylasparagine*—4 gm. of the above bromo ester, dissolved in 10 cc. of absolute methanol, were added to a methanol solution of the methyl ester of glycylasparagine which had been prepared from 3.8 gm. of free glycylasparagine.[1] The mixture was brought to pH 8 with sodium methoxide, concentrated to 20 cc., and allowed to stand overnight at room temperature. 10 cc. of aqueous 4 N NaOH and 30 cc. of methanol were added, and after 1 hour excess alkali was neutralized with acetic acid. A solution of 10 gm. of barium acetate in 100 cc. of water was added, and the concentration of methanol was adjusted to 70 per cent. The barium salts were allowed to separate overnight in the cold, and they were then collected, washed, and dissolved in 150 cc. of water and reprecipitated with 450 cc. of methanol. They were finally dissolved in water, freed of barium ion exactly with sulfuric acid, and the solution so obtained was concentrated under reduced pressure below 40° to a small volume. Addition of alcohol produced a white precipitate, which was allowed to form for several days in the cold and was then collected and washed. 410 mg. of substance were obtained which were purified by solution in water and reprecipitation with alcohol.

$C_9H_{15}O_7N_3$. Calculated, N 15.2; found, N 15.5

One-third of the nitrogen was amide nitrogen which was liberated as ammonia during hydrolysis with 1 N HCl. Found, 5.25; calculated, 5.07. The substance gave many of the reactions shown by lycomarasmin, such as the iodoform test and the formation of a white, highly insoluble precipitate when the dry material was suspended in 1 N NaOH. It was an acid which dissolved readily in NaOH and did not give this white precipitate if a large excess of alkali was avoided. The material differed from natural lycomarasmin in that it was more soluble in water. Distinctive derivatives of lycomarasmin are lacking, so that precise comparison was not possible. The synthetic substance had the same biological potency as natural lycomarasmin, whereas glycylasparagine and glycylaspartic acid gave no effect at 25 times the concentration.

---

[1] This ester was prepared by suspending 3.8 gm. of glycylasparagine in 50 cc. of anhydrous methanol which was then saturated with dry HCl. 100 cc. of methanol were added and the mixture was held at 4° for 2 days. The solvent was then removed under reduced pressure at 4° and excess HCl was eliminated by several additions of methanol, followed by distillation under reduced pressure. The product was then dried in a vacuum desiccator over KOH. The free ester was obtained by suspension of the hydrochloride in methanol and addition of the theoretical amount of sodium methoxide dissolved in methanol. Sodium chloride was removed by filtration.

Biological Assay with Tomato Leaves—The synthetic substance was assayed for ability to cause wilting and curling of excised tomato leaves, and it was found that 0.2 mg. per 7 cc. of test medium was the minimal effective amount. Lycomarasmin showed the same value in a series of parallel assays. The conditions of the test were those described by Clauson-Kaas *et al.* (1, 2). In order to improve the accuracy of the test, it was found important to select leaves of uniform size and age and to conduct the experiments in a room provided with controlled illumination and with constant temperature and humidity (50 per cent). Graded 2-fold dilutions of the material under test were supplied to the leaves, and the results were read after 40 hours. Severity of wilting and curling was then judged, and the minimal effective dose which would just cause detectable alteration of the leaves was taken as the end-point. Activity was not estimated solely by the minimal effective dose, but rather by the responses to larger amounts as well. The synthetic and natural products seemed to be equal in potency.

SUMMARY

Lycomarasmin, a tomato leaf-wilting toxin isolated by Plattner and Clauson-Kaas from culture filtrates of the phytopathogenic fungus *Fusarium lycopersici*, has been studied in an effort to arrive at a satisfactory chemical structure for it. This small, peptide-like toxin, of empirical formula $C_9H_{15}O_7N_3$, which yielded glycine and aspartic acid on hydrolysis, was shown to be a derivative of asparagine. Thus the 3rd N atom, which had previously not been assigned position, was located. Because of the reactions which lycomarasmin underwent, a structural formula for it was proposed which contained the new amino acid, α-hydroxyalanine. This was attached by a common nitrogen atom (*i.e.*, the amino group) to the amino group of glycylasparagine. A synthetic product was isolated from the reaction of ethyl-α-acetoxy-α-bromopropionate and the methyl ester of glycylasparagine, which had properties in common with lycomarasmin, including quantitatively the same biological activity. Some reasons were advanced for regarding lycomarasmin as a much simplified model of proteins.

BIBLIOGRAPHY

1. Plattner, P. A., and Clauson-Kaas, N., *Helv. chim. acta*, **28**, 188 (1944).
2. Clauson-Kaas, N., Plattner, P. A., and Gäumann, E., *Ber. schweiz. bot. Ges.*, **54**, 523 (1944).
3. Plattner, P. A., and Clauson-Kaas, N., *Experientia*, **1**, 195 (1945).
4. Woolley, D. W., *J. Biol. Chem.*, **172**, 71 (1948).
5. Woolley, D. W., *J. Biol. Chem.*, **171**, 443 (1947).
6. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **122**, 419 (1937–38).
7. Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, **38**, 224 (1944).
8. Hac, L. R., and Snell, E. E., *J. Biol. Chem.*, **159**, 291 (1945).
9. Shankman, S., Camien, M. N., and Dunn, M. S., *J. Biol. Chem.*, **168**, 51 (1947).

SYNTHESIS AND DETERMINATION OF LYCOMARASMIN ACTIVITY OF SOME DERIVATIVES OF ASPARTIC ACID

By D. W. WOOLLEY*

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, August 6, 1948)

Since the phytopathogenic toxin of *Fusarium lycopersici*, first isolated by Plattner and Clauson-Kaas (1), has been shown by Woolley (2) probably to have the structure of N-(α-(α-hydroxypropionic acid))-glycylasparagine, the synthesis and determination of the activity of a number of closely related compounds became of interest. Not only would such a study add to our knowledge of the relationship of structure to biological activity, but more especially it would allow investigation on a much simplified scale of some problems concerned with the specific activities of proteins. Since lycomarasmin is a very small peptide with marked activity, the determination of how its biological properties are altered by rearrangement of the constituent amino acids, or by substitution of new groups, might serve as a useful starting point for understanding of similar phenomena connected with the specificity of protein action. Therefore, a number of new compounds related to lycomarasmin, as well as some previously known ones, have been prepared and tested for their ability to cause wilting and curling of excised tomato leaves. Their potency in this regard has been compared with that of the natural toxin.

EXPERIMENTAL

Sources of Known Compounds—Lycomarasmin was isolated from culture filtrates of *Fusarium lycopersici* according to the directions of Plattner and Clauson-Kaas (1). Aspartic acid, asparagine, serine, glycine, and glutathione were commercial samples. Pyruvylglycine was made according to the directions of Bergmann and Grafe (3), and glycylaspartic acid and glycylasparagine by modifications of published methods (4). These modifications were the use of excess of aspartic acid or of asparagine in the reactions with chloroacetyl chloride, and the removal of traces of chloride ions from the glycyl compounds with silver acetate. By using an excess of the amino acid during the acylation, contamination of the product with chloroacetic acid was minimized. The chloroacetylaspartic acid was extracted into ethyl acetate from aqueous solution, and then readily crystallized. The chloroacetylasparagine was not extractable with this solvent, so that the reaction mixture was first purified by removal of ethyl acetate-soluble matter and then by evaporation of the aqueous phase and solution of the desired product in acetone. It was finally purified by crystallization according to published methods. Syntheses of serylglycylaspartic acid, serylglycylglutamic acid, glycylserylglutamic acid, and alanylglycylglutamic acid have been described previously (5, 6). Serylglycylaspartic acid was a diastereomeric mixture prepared from DL-serine and L-aspartic acid. In addition, a sample was made from DL-serine and DL-aspartic acid in order to compare the relative potencies of the two pairs of isomers.

---

* With the technical assistance of R. Brown and A. Holloway.

Glycylserylaspartic Acid—Tosylglycyl-DL-serine azide was coupled with diethyl-L-aspartate, and the free peptide was formed in the manner described for the isomeric serylglycylaspartic acid (6).

$C_9H_{15}O_7N_3$. Calculated, N 15.2; found, N 15.0

The product was a mixture of diastereomers, since DL-serine had been used.

Glycylaspartylserine. (*a*) *Chloroacetylaspartylserine*—Chloroacetyl-L-aspartic acid was converted to the anhydride by dissolving 16.8 gm. of it in 100 cc. of acetic anhydride and refluxing the solution for 2 minutes. It was then concentrated under reduced pressure to a sirup, which was caused to crystallize by addition of 10 cc. of dry chloroform. Precipitation was completed by further addition of 50 cc. of absolute ether. The anhydride so obtained was converted to the monoethyl ester by dissolving it in 50 cc. of hot absolute ethanol, cooling, and allowing the solution to stand for 24 hours at room temperature. The monoester was purified by removing the alcohol under reduced pressure, dissolving the oily residue in absolute ether, and extracting the latter solution with 150 cc. of ice-cold 5 per cent aqueous $NaHCO_3$ solution. The acidic half-ester in the aqueous phase was quickly liberated with 1 equivalent of HCl and extracted into fresh ether. This ether extract was then dried rapidly with $MgSO_4$, filtered, and freed of solvent under reduced pressure. It was thus secured as 8.9 gm. of a colorless oil. By analogy with the behavior of the corresponding tosyl derivative (7), it was probably mainly the α, rather than the β ester.

The chloroacetylmonoethylaspartate obtained as described above was dissolved in 100 cc. of absolute ether and treated with 9.0 gm. of PCl_5 for about 30 minutes. The clear solution was then decanted, and the ether was removed under reduced pressure at low temperature. The $POCl_3$ was extracted from the residue with cold (−10°) dry petroleum ether, whereupon the acid chloride crystallized as large rosettes. Without further purification, it was suspended in 100 cc. of absolute ether, and to the solution were added 5.2 gm. of DL-serine dissolved in 60 cc. of ice-cold 1 N NaOH. The mixture was shaken vigorously and maintained faintly pink to phenolphthalein by frequent additions of cold 4 N NaOH. The reaction was allowed to proceed for an hour in the cold, and then the mixture was acidified to pH 2.5 with HCl, and the ether layer was discarded. The aqueous phase was extracted four times with ethyl acetate, and the extracts were discarded. The desired product was then obtained by extracting the aqueous layer five times with butanol, and the combined extracts were dried with silica gel, filtered, and washed twice with 10 cc. of water. The peptide was then extracted into aqueous solution with enough NaOH to give a final pH of 7. Mild alkaline hydrolysis of an aliquot of this solution, followed by back titration, showed that some ethyl ester of the required peptide probably still remained mixed with the free acid. Therefore 10 cc. of 1 N NaOH were added, and, after 1 hour, this was acidified with 1 equivalent of HCl. The reaction mixture was then concentrated under reduced pressure at a temperature not above 30° to a small volume and treated with 10 volumes of acetone. The acetone-soluble portion yielded 3.5 gm. of an oil, which was caused to crystallize by cautious addition of ether.

$C_9H_{13}O_7N_2Cl \cdot H_2O$. Calculated, N 8.9; found, N 8.9

(*b*) *Glycylaspartylserine*—3.5 gm. of the compound just described were dissolved in 250 cc. of concentrated aqueous ammonium hydroxide, and after the solution had stood 3 days at room temperature, it was concentrated under reduced pressure to about 10 cc. and treated with 200 cc. of absolute ethanol. The solid which was thus produced was centrifuged and washed with alcohol. In order to free it of chloride ions, it was dissolved in water, treated with an excess of silver acetate, and the filtrate from the mixture was freed of silver ions with H_2S. When the concentrated aqueous solution of the product was treated with alcohol, a flocculent precipitate (2.1 gm.) was formed, which was too high in N to be the desired tripeptide. Therefore, it was dissolved in water, brought to pH 8 with barium hydroxide, and the barium salt was crystallized by addition of 2 volumes of methanol. When barium ions were exactly removed from this insoluble salt with sulfuric acid, the free peptide was precipitated as a white powder when alcohol was added to a concentrated aqueous solution of it.

$C_9H_{15}O_7N_3$. Calculated. N 15.2, aspartic acid 48
Found. " 15.1, " " 47

Aspartic acid was estimated microbiologically according to the directions of Hac and Snell (8), following acid hydrolysis of the peptide.

Acetyllactylglycylasparagine—9.5 gm. of glycyl-L-asparagine, prepared as indicated above, were acylated in cold, alkaline aqueous solution with an ether solution of 15 gm. of acetyllactyl chloride. The conditions of the condensation were like those described in the preceding section for the acylation of serine. After a reaction time of 1 hour, the mixture was acidified to pH 2.5 with HCl, and the aqueous phase was extracted three times with ether and three times with ethyl acetate. The extracts were discarded, and the aqueous phase was concentrated under reduced pressure, and below 40°, to a sirup. This was extracted with 500 cc. of acetone, and the soluble portion was concentrated to a thin sirup which was extracted with a few cc. of acetone. This operation caused the separation of a gummy precipitate which was collected and dissolved in 40 cc. of methanol. When chloroform was added to this solution, crystallization began. A yield of 13.8 gm. of hygroscopic, large crystals was obtained.

$C_{11}H_{17}O_7N_3$. Calculated, N 13.9; found, N 13.9

This compound was prepared as a starting material for the synthesis of pyruvylglycylasparagine, for it was hoped to remove the acetyl group by mild alkaline hydrolysis and then to oxidize the secondary alcohol to a ketone. Although the cleavage of the acetyl group seemed to proceed readily at room temperature in a slight excess of aqueous NaOH, the product obtained was amorphous and very hygroscopic. For these reasons a satisfactory preparation could not be made. Attempted oxidation of the hydrolyzed product with Fenton's reagent for the formation of the pyruvyl compound gave no desired substance which could be purified satisfactorily.

α,α-Diacetaminopropionylglycylasparagine—8.5 gm. of the azlactone of α,α-diacetaminopropionic acid (3) were suspended in 150 cc. of cold alcohol and immediately mixed with an ice-cold solution of 9.5 gm. of glycyl-L-asparagine in 52 cc. of 1 N NaOH. The resulting solution was kept at room temperature for 30 minutes and then acidified with 1 N H_2SO_4 equivalent to the NaOH used, and concentrated under reduced pressure to dryness below 40°. The residue was dried by adding 100 cc. of absolute alcohol and again concentrating it under reduced pressure. It was then extracted with 400 cc. of boiling ethanol, and the mixture was filtered hot. A slight precipitate which formed when the filtrate was cooled was discarded, and the alcoholic solution was concentrated under reduced pressure to about 30 cc. and treated with 100 cc. of ethyl acetate. 10 gm. of the product crystallized, and this was recrystallized by adding ethyl acetate to an alcoholic solution. The purified material sintered at 140° and melted at 185–187°.

$C_{13}H_{21}O_7N_5$. Calculated. C 43.5, H 5.9, N 19.5
Found. " 43.5, " 6.2, " 19.0

The neutral equivalent was 370, while the calculated value was 359.

α-Hydroxy-α-acetaminopropionylglycylaspartic Acid—1 gm. of diacetaminopropionylglycylasparagine was dissolved in 100 cc. of 0.2 N HCl, and the solution was refluxed for an hour and cooled. It was then adjusted to pH 8 with barium hydroxide, treated with 3 volumes of methanol, and kept in the cold overnight. The precipitate which had formed was collected and washed well, and then dissolved in water for the exact removal of barium ions with sulfuric acid. The resulting aqueous solution was concentrated under reduced pressure below 40° to a sirup, which was extracted with alcohol in order to free it of pyruvylglycylaspartic acid (75 mg.). The alcohol-soluble portion was freed of solvent, dissolved in water, and adjusted to pH 8 with barium hydroxide. The addition of 2 volumes of methanol and storage in the cold produced a network of crystals, and these were collected, washed, and dried; yield, 264 mg.

$C_{11}H_{15}O_8N_3Ba \cdot H_2O$. Calculated. C 28.0, H 3.6, N 8.9, Ba 29.2
Found. " 25.2, " 3.4, " 8.4, " 30.7

The water of hydration was not proved to exist by analysis. Although the analytical results indicated that the salt was not pure, the ratios between the elements showed clearly that a barium salt of a dibasic acid was the major component. The free acid was regenerated by solution of the salt in water and addition of an exact equivalent of H_2SO_4. The aqueous solution was freed of $BaSO_4$ and allowed to evaporate slowly in a desiccator over KOH. After many days, crystals were obtained which melted at 119–120°.

$C_{11}H_{17}O_8N_3$. Calculated. C 41.4, H 5.3, N 13.2
Found. " 42.0, " 5.1, " 13.0

The crystals were relatively unstable and became oily when stored at room temperature for several months, possibly due to dehydration of the alcoholic group.

This substance as it was obtained in this work was quite probably a mixture of diastereomeric compounds, because, during the hydrolysis, a new asymmetric carbon atom was produced. Since the aspartic acid residue was optically active, the appearance of a second asymmetric center should give a mixture of two isomers, and these may not have been separated during the purification. No special attempt was made to part this mixture.

The question may well be raised as to whether the tertiary alcoholic group actually was present in the propionic acid residue of this substance, or whether it had been eliminated as water, leaving an acrylic acid residue. Although there was no direct evidence on this point, the analytical values agreed better with the postulate that the hydroxyl group remained than with the idea of an acrylic acid compound. The high lycomarasmin activity of the compound (which will be described below) fitted into the belief that the hydroxyl still remained, because such a structure would contain the substituted α-hydroxyalanine residue which is found in lycomarasmin (2).

Pyruvylglycylaspartic Acid—As has already been indicated, this compound was obtained in small yield as an alcohol-insoluble by-product during the preparation of the previously described substance. It was isolated in greater amounts by increasing the rigor of the hydrolysis. Thus, 1 gm. of α,α-diacetaminopropionylglycylasparagine in 100 cc. of 1 N H_2SO_4 was heated to 100° for an hour, and the barium salts insoluble in 75 per cent methanol were taken and freed of barium in the usual fashion. Concentration of the aqueous solution of the free acid to a sirup, followed by addition of alcohol, gave crystals which were purified by solution in NaOH at pH 7 and acidification with 1 equivalent of HCl. The substance was sparingly soluble in water or in alcohol, but when impure it crystallized from these solvents only very slowly.

$C_9H_{12}O_7N_2$. Calculated, N 10.7; found, N 10.8

The neutral equivalent was found to be 126, whereas the theoretical value was 130.

This compound was isomeric with the degradation product of lycomarasmin which Plattner and Clauson-Kaas (1) have described as Substance I. The rather close resemblance of pyruvylglycylaspartic acid to Substance I led us to consider them to be identical until subsequent work, especially with the iodoform reaction (2), showed this not to be true.

α,α-Diacetaminopropionylglycylaspartic Acid—This compound was prepared from glycyl-L-aspartic acid in a manner analogous to that described previously for the corresponding asparagine derivative. It sintered at 103° and melted at 160–165°.

$C_{13}H_{20}O_8N_4$. Calculated, N 15.6; found, N 15.4

By mild acid hydrolysis this compound was converted into pyruvylglycylaspartic acid, which was the same as that from α,α-diacetaminopropionylglycylasparagine.

Method of Biological Testing with Tomato Leaves—Assays for activity in causing excised tomato leaves to wilt and curl were conducted in a manner already described for lycomarasmin (1, 2). The reproducibility of the results of the test was increased materially by attention to the details already enumerated (2), particularly the use of leaves of uniform age and size and the maintenance of constant humidity. Graded dilutions of each substance to be tested were mixed with $FeCl_3$ (final concentration 0.1 mg. per 7 cc.), and each dilution was tested on several individual tomato leaves. All potencies were estimated relative to that of natural lycomarasmin, and therefore, in each assay, a series of dilutions of this standard was included. The pH of all solutions was adjusted to 7 before the leaves were exposed to them.

The activity of each substance tested was judged by noting the minimal concentration which would just cause detectable wilting or curling in a majority of the leaves, and also by judging the amount which would cause maximal effect. In doing these estimations some errors of judgment occur, because even with lycomarasmin, a single concentration may cause more

TABLE I

Responses of Tomato Leaves to Graded Amounts of Lycomarasmin

Lycomarasmin	Severity of wilting and curling
mg. per 7 cc.	
0.1	0
0.2	+
0.3	++
0.4	+++
0.8	++++
2.0	++++

TABLE II

Amounts of Various Compounds Necessary for Wilting and Curling Response in Excised Tomato Leaves

Compound	Detectable at	Maximum effect at
	mg. per 7 cc.	*mg. per 7 cc.*
Lycomarasmin	0.2	0.8
L-Asparagine	5	>30
L-Aspartic acid	15	>30
Glycine	5	>30
Pyruvylglycine	Not detectable at 6	
DL-Serine	5	>30
Glycylasparagine	15–30	
Glycylaspartic acid	Not detectable at 12	
Serylglycylaspartic acid from L-aspartic acid*	4–6	8–14
" " " DL-aspartic "	4–6	12
Glycylserylaspartic acid*	2–4	6
Glycyl-β-aspartylserine	No effect at 14	
Acetyllactylglycylasparagine	8–12	
Diacetaminopropionylglycylasparagine	4	>14
Diacetaminopropionylglycylaspartic acid	Not detectable at 15	
α-Hydroxy-α-acetaminopropionylglycylaspartic acid	0.3	4
Pyruvylglycylaspartic acid	Not detectable at 3	
Serylglycylglutamic acid	" " " 7	
Glycylserylglutamic acid	" " " 6	
Alanylglycylglutamic acid	" " " 7	
Glutathione	" " " 6	

---

* The activities of these two compounds, which were given in a preliminary note (5), made them appear too active relative to lycomarasmin, because it was found subsequently that the lycomarasmin which had been used for comparison was impure.

wilting than curling in some individual leaves and more curling than wilting in others. To arrive at a value, each leaf was rated on a scale ranging from

± to ++++, by taking into account the extent of wilting, of curling and shriveling, and of obvious necrosis. Between the extremes of ± and ++++ there was a range in which graded amounts produced graded severity of change in the leaves. This is illustrated by the data in Table I. The ratings of individual leaves in a group exposed to the same concentration of a substance were usually in good agreement. The values for the activity of a compound could be duplicated by subsequent assays within a factor of 2. Frequently identical values were found in such duplicate determinations.

Lycomarasmin Activity of Various Compounds—The lycomarasmin activity of a number of substances was determined in the manner just indicated, and the results are summarized in Table II.

DISCUSSION

Among the compounds tested, it is evident that small changes in the nature of the amino acid residues had a greater influence on activity than did slight changes in the position of these residues. In general, even minor changes in the amino acid composition affected potency very much, while considerable alteration in the manner of linkage of the amino acids usually had a minor influence. Lycomarasmin may be viewed as a substance containing 3 amino acid residues; *viz.*, those of asparagine, glycine, and α-hydroxyalanine. When these three components were maintained, but rearranged so that the α-hydroxyalanine was attached to glycine in a peptide linkage rather than by a common nitrogen atom (as is the case in lycomarasmin), a relatively active compound, α-hydroxy-α-acetaminopropionylglycylaspartic acid, was realized, even despite the fact that it was a derivative of aspartic acid rather than of asparagine, and that its amino group was covered with an acetyl radical. However, when the amino acid nature of this substance was changed by moving the hydroxyl to the β position, as in serylglycylaspartic acid, the activity was diminished appreciably. Furthermore, when the change was greater and the amino group eliminated, as in acetyllactylglycylasparagine, the resulting compound was much less active than lycomarasmin. The acetyl group in the acetyllactylglycylasparagine did not seem to be particularly inimical to potency, because removal of it by mild alkaline hydrolysis did not greatly increase the biological activity. The relative potencies of serylglycylaspartic acid and of serylglycylglutamic acid throw more light on the effect of changing slightly the amino acid composition. The mere introduction of a CH_2 grouping in passing from aspartic to glutamic acid was enough to reduce the potency of the compound. Other similar examples may be seen from the data in Table II.

In considering what features of the lycomarasmin molecule may be responsible for its biological activity, one should note two points from this study. The first is that in the three pairs of similar compounds which differed only in that one of the set was an aspartic acid derivative while the other was from asparagine, the asparagine compounds were slightly more active.[1] The second is the rise and fall of activity as one passed, by partial hydrolyses, from diacetaminopropionylglycylasparagine, through α-hydroxy-α-acetaminopropionylglycylaspartic acid to pyruvylglycylaspartic acid. Maximal potency was displayed in this series by the compound containing an α-hydroxyalanine residue. It will be remembered that lycomarasmin contained both an asparagine and an α-hydroxyalanine residue, while inactive Substance I obtained from it by heating, possessed neither.

The effect of changing positions of the amino acid residues may be studied in one of its aspects from the results with the three peptides containing serine, glycine, and aspartic acid. Serylglycylaspartic acid and glycylserylaspartic acid were approximately of the same activity. However, glycylaspartylserine was inactive. One may argue that the placing of a serine residue on the β-carboxyl group of aspartic acid has blocked the possibility of amidation to asparagine which might take place in the leaves during the test, but nevertheless the inactivity of this position isomer should serve to show the dangers and limitations of generalizations about the relative effects of changing composition compared to altering arrangement of the amino acid residues. Many sorts of isomers must be examined before the question can be answered, even for a simple peptide such as lycomarasmin.

The results with the two preparations of serylglycylaspartic acid, one made from L-aspartic acid and the other from the DL isomer, would tend to suggest that optical configuration is not a crucial matter in lycomarasmin activity. Although the two preparations were admittedly mixtures of diastereomers, which may have been partially separated during the purification procedures, the results were essentially the same when the assays were performed on the crude reaction mixtures of the synthesis, before any purification had taken place.

The large number of substances which showed some ability to wilt and curl tomato leaves must raise a question about the specificity of the test for lycomarasmin. In our experience, almost any substance, even NaCl or NH_4Cl, will cause some wilting of the leaves if the concentration is high enough. This is probably due to disturbances of osmotic pressure, or to other changes, and not to those effects associated with the action of lycomarasmin. Gäumann and Jaag (9) have examined the characteristics of the alterations in leaves attributable to the toxin and have compared them to those found in leaves wilted from other causes. However, at present no way of improving the specificity of the lycomarasmin test seems evident.

[1] Both members of the pair glycylasparagine and glycylaspartic acid were so little active as to make their contribution to this point questionable.

Possibly one qualitative aid might be the extensive and severe character of the changes elicited by the toxin in comparison to those of substances such as glycine or aspartic acid. In these latter cases the production of black, necrotic spots and the drying and shriveling of the leaf edges were not evident as they were with lycomarasmin and with the more active compounds studied in this investigation. The interpretation to be placed on the activities of compounds less than 5 per cent as effective as lycomarasmin must be open to grave doubts. In fact, the biological relationship of any of the substances to the natural toxin will remain open to question until a more specific test can be evolved.

SUMMARY

A number of new derivatives of aspartic acid and of asparagine have been prepared in order to examine the effects of various structural changes on the activity of lycomarasmin, which is a peptide derived from asparagine, glycine, and probably α-hydroxyalanine. Thus, glycylserylaspartic acid, glycyl-β-aspartylserine, acetyllactylglycylasparagine, α,α-diacetaminopropionylglycylasparagine, the corresponding aspartic acid compound, α-hydroxy-α-acetaminopropionylglycylaspartic acid, and pyruvylglycylaspartic acid were synthesized. These, along with known compounds such as glycylaspartic acid, glycylasparagine, aspartic acid, asparagine, glycine, serine, pyruvylglycine, serylglycylaspartic acid, serylglycylglutamic acid, and glutathione, were compared with lycomarasmin for ability to cause excised tomato leaves to wilt and curl. Several of the peptides were somewhat less active in this respect than was lycomarasmin, but one of them, α-hydroxy-α-acetaminopropionylglycylaspartic acid, was about equal to the natural toxin in activity. Some produced no detectable effect. Comparison was made of the relative effect on activity of changing the position of an amino acid in a peptide with that of altering the nature of the amino acid. From the limited data, changes in position seemed to affect potency less than replacement by a new amino acid, but the limitations of the argument were recognized. The specificity of the biological test was also discussed.

BIBLIOGRAPHY

1. Plattner, P. A., and Clauson-Kaas, N., *Helv. chim. acta*, **28**, 188 (1944).
2. Woolley, D. W., *J. Biol. Chem.*, **176**, 1291 (1948).
3. Bergmann, M., and Grafe, K., *Z. physiol. Chem.*, **187**, 187 (1930).
4. Fischer, E., and Koenigs, E., *Ber. chem. Ges.*, **37**, 4585 (1904).
5. Woolley, D. W., *J. Biol. Chem.*, **166**, 783 (1946).
6. Woolley, D. W., *J. Biol. Chem.*, **172**, 71 (1948).
7. Bovarnick, M. R., *J. Biol. Chem.*, **148**, 151 (1943).
8. Hac, L. R., and Snell, E. E., *J. Biol. Chem.*, **159**, 291 (1945).
9. Gäumann, E., and Jaag, O., *Ber. schweiz. bot. Ges.*, **57**, 227 (1947).

THE INFLUENCE OF PTEROYLGLUTAMIC ACID ON NUCLEIC ACID SYNTHESIS IN LACTOBACILLUS CASEI*

By W. H. PRUSOFF, L. J. TEPLY, and C. G. KING

(*From the Department of Chemistry, Columbia University, New York*)

(Received for publication, July 24, 1948)

Several findings reported in the literature point toward a functional relationship between pteroylglutamic acid (PGA, vitamin B_c, folic acid) and the synthesis of certain constituents of nucleic acids, but the related specific reactions have not been identified. Snell and Mitchell (1) observed that thymine plus a purine base could partially replace PGA for *Streptococcus faecalis* R in a medium similar to that used by Snell and Peterson (2) in their report on the *Lactobacillus casei* factor. Stokstad (3) observed a similar relationship for *Lactobacillus casei*, but only about one-half maximum acid production could be induced by the substituted nutrient. Stokes (4) suggested that PGA may "function as a coenzyme for the enzyme system responsible for the synthesis of thymine (or more probably a thymine-like compound) which, in turn, is used by the bacteria to form nucleic acid."

Observations by others, however, suggested that PGA is not involved directly in the synthesis of thymine. Hitchings, Falco, and Sherwood (5) studied the effect of pyrimidine derivatives on *Lactobacillus casei*. The organism was inhibited by 5-bromouracil when grown in media containing thymine, but was not inhibited in the presence of PGA. In contrast, 5-nitrouracil inhibited growth when PGA was supplied but not when thymine was substituted. This reversal was interpreted as indicating that thymine and folic acid were not components of the same anabolic system. Hall (6) observed a synergistic effect between thymine and PGA in the growth of *Streptococcus faecalis* R and suggested that "thymine may actually be a precursor of folic acid or that thymine is participating in some alternate metabolic path." Strandskov and Wyss (7) observed that an analogue of thymine, thiothymine, competitively inhibited *Lactobacillus casei* in the presence of thymine, but was inactive in the presence of PGA. This was given as "strong evidence that thymine precedes B_c in some synthetic process." Alternate metabolic pathways for thymine and folic acid could also explain the effect of thiothymine.

Inhibition studies by Lampen and Jones (8, 9) with sulfonamides and by Rogers and Shive (10) with a folic acid analogue, methylfolic acid, indi-

---

* This investigation was supported by a grant from the Nutrition Foundation, Inc.

cate that PGA is involved in the biosynthesis of thymine, or a thymine-like compound, and purines. Stokstad *et al.* (11) observed additional interrelationships in cultures of *Streptococcus faecalis* R, indicative that PGA may be involved in the synthesis of thymine and adenine. Stetten and Fox (12) observed, with *Escherichia coli* during bacteriostasis due to sulfonamides, an accumulation of an amine which was identified by Shive *et al.* (13) as 5(4)-amino-4(5)-imidazolecarboxamide. This compound may be either a purine precursor or formed from a purine precursor. Formylfolic acid or rhizopterin has been suggested as a formylating agent in the introduction of a carbon unit into this amine. Schopfer (14) observed that bacteriostasis by sulfathiazole could be offset in part by adenine or by nucleic acids.

In the present investigation, an impairment in the synthesis of desoxyribonucleic acid (DNA), but not of ribonucleic acid (RNA), was found to result regularly from PGA deficiency.

EXPERIMENTAL

Culture and Medium—The organism used was *Lactobacillus casei* 7469. Monthly transfers were made as stab cultures in a medium containing 1.5 per cent of Bacto-agar, 0.5 per cent of glucose, and 2 per cent of Bacto-yeast extract. After incubation at 37° for 22 hours, the cultures were stored at approximately 5°.

The inoculum was prepared first by a transfer from the stock culture to a tube containing the above medium. After incubation for 18 to 22 hours, a second transfer was made into a medium containing 0.5 per cent of glucose and 2 per cent of Bacto-yeast extract. The cells were centrifuged after 22 hours and washed three times with sterile 0.9 per cent sodium chloride solution. Sterile saline was added to the suspension of cells until a standard density reading was obtained (89 to 90 in an Evelyn photocolorimeter, Filter 720). A 2.5 ml. aliquot of this suspension was used to inoculate 500 ml. of the medium described in Table I.

The medium was essentially that of Teply and Elvehjem (15), with the vitamin modification of Roberts and Snell (16). Half liter quantities were sterilized in 1 liter Erlenmeyer flasks for 15 minutes at 20 pounds pressure. Upon removal from the autoclave, the flasks were cooled in an ice bath.

Harvesting of Cells—After incubation at 37° for 22 hours, the inoculated flasks were cooled to 0–5° and centrifuged under refrigeration. The cells were washed once with ice-cold distilled water (volume approximately × 20) and adjusted to volume (100 to 250 ml.).

Methods of Analysis—Aliquots of the bacterial cell suspensions were used for the following analyses:

Dry Weight—Triplicate samples were dried at 105° for 12 hours.

Nitrogen—Duplicate samples were digested according to the method of Koch and McMeekin (17). After appropriate dilution, 1.5 ml. of modified Nessler-Folin reagent (17) were added to 10 ml. of the diluted sample. Light transmission was read in an Evelyn photoelectric colorimeter, Filter 420.

Acidity—10 ml. aliquots of the supernatant of the medium were electrometrically titrated with 0.1 N KOH to pH 7.2.

TABLE I

Composition of Media

Component	Quantity per liter	Component	Quantity per liter
Glucose (H_2O)	22 gm.	Choline	2.5 mg.
Casein*	5.0 "	Salts B†	5.0 ml.
K_2HPO_4	2.5 "	*p*-Aminobenzoic acid	100 γ
Sodium acetate ($3H_2O$)	33.2 "	Calcium pantothenate	500 "
L-Asparagine	100 mg.	Nicotinic acid	500 "
DL-Alanine	200 "	Pyridoxine hydrochloride	1000 "
L-Cystine	200 "	Thiamine hydrochloride	500 "
DL-Tryptophan	400 "	Biotin‡	4 "
Adenine sulfate	10 "	Riboflavin§	500 "
Guanine hydrochloride	10 "	Pteroylglutamic acid‖	2.0 "
Uracil	10 "	Thymine¶	500 "
Xanthine	10 "		

* Squibb's casein enzymatic hydrolysate, treated four times with 10 per cent by weight of Darco G-60 carbon at pH 3.0 for 0.5 hour periods.

† Salts B = $MgSO_4 \cdot 7H_2O$ 4.0 gm., NaCl 0.2 gm., $FeSO_4 \cdot 7H_2O$ 0.2 gm., $MnSO_4 \cdot 4H_2O$ 0.2 gm., H_2O to 100 ml., plus 0.5 ml. of 50 per cent H_2SO_4.

‡ Biotin-restricted medium contained 0.1 γ per liter.

§ Riboflavin-restricted medium contained 10 γ per liter.

‖ Pteroylglutamic acid-restricted medium contained 0.05 γ per liter. We are indebted to Dr. T. H. Jukes of the Lederle Laboratories Division, American Cyanamid Company, for supplying generous quantities of PGA.

¶ Thymine was included only when pteroylglutamic acid was omitted.

Turbidity—Transmission was measured in an Evelyn photoelectric colorimeter with Filter 720, adjusted to read 100 when water was used.

Nucleic Acids—Duplicate samples were used for the extraction of nucleic acids by the method of Schneider (18). The bacterial cells were treated with 7 per cent trichloroacetic acid in an ice-water bath and stirred every 15 minutes through a 2 hour period. The suspension was then centrifuged under refrigeration and washed once with 20 times its volume of ice-cold 5 per cent trichloroacetic acid. The residue was treated with 40 times its volume of 5 per cent trichloroacetic acid and heated for 15 minutes at 90°, with frequent stirring. After being centrifuged, the residue was reheated with 5 per cent trichloroacetic acid for 15 minutes at 90°. The supernatants from the two extractions were combined and made up to volume.

Desoxyribonucleic acid was determined by the diphenylamine reaction described by Dische (19). The desoxyribonucleic acid standard was prepared from fresh calf thymus glands by the modified method of Levene and Bass (20).

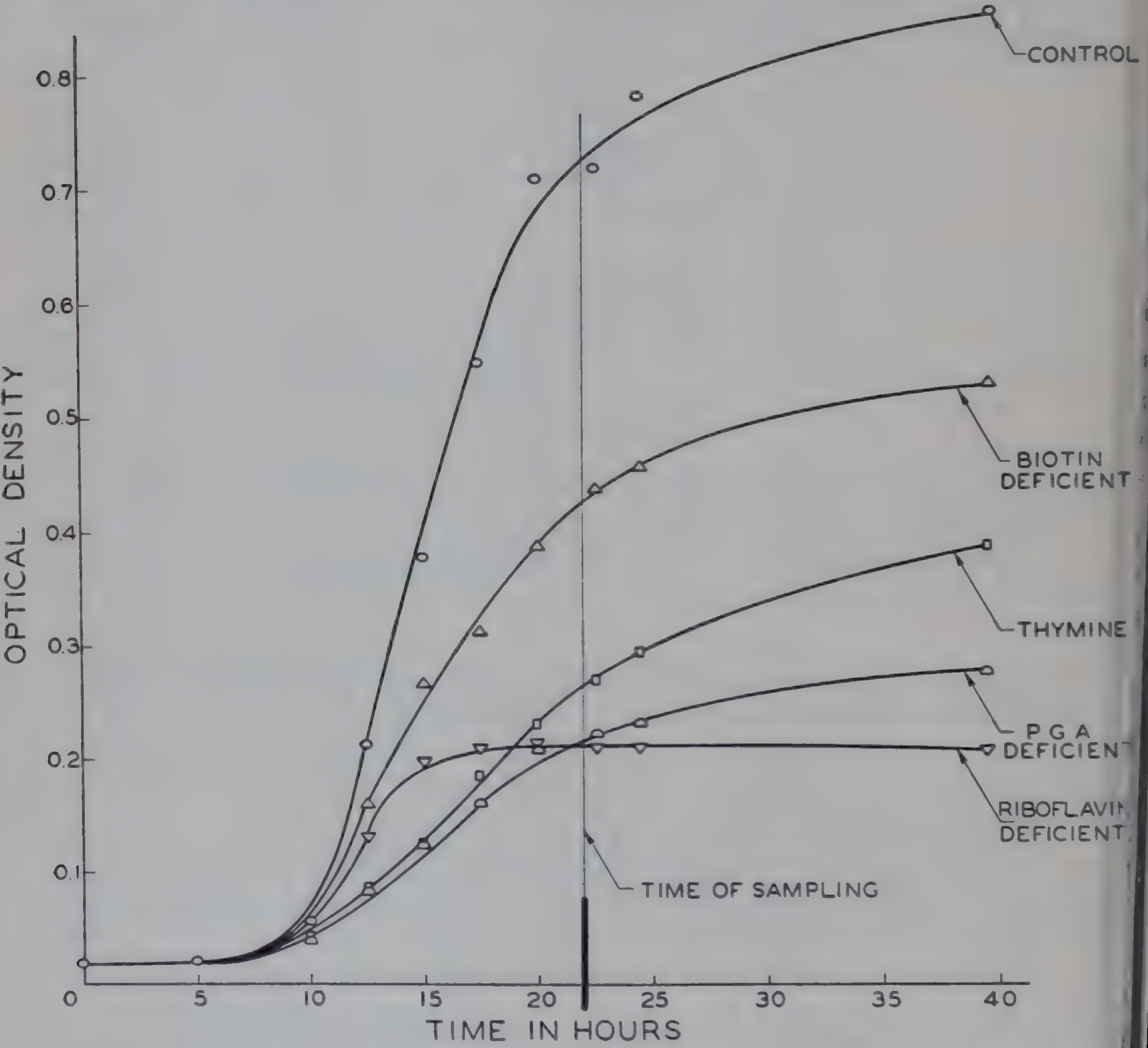


FIG. 1. Rate of growth of *Lactobacillus casei* in media containing excess PGA (control), thymine substituted for PGA, and deficient in PGA, riboflavin, or biotin.

Ribonucleic acid was determined by the Albaum and Umbreit modification (21) of the orcinol reaction (22). A correction (18) for the desoxyribonucleic acid content was applied. The ribonucleic acid standard was a yeast preparation obtained from Merck and Company.

The purity of both standards was estimated from their phosphorus content compared to that calculated for a tetranucleotide structure. On this basis, RNA and DNA were 88.4 and 87.1 per cent pure, respectively. The reported nucleic acid values included consideration of the purity correction applied to the standard nucleic acids. The N:P ratios for RNA and DNA were 1.68 and 1.60, respectively, compared to the calculated ratios of 1.70 and 1.69. Choice of a proper nucleic acid standard is difficult, because of incomplete information regarding composition of the pure acids.

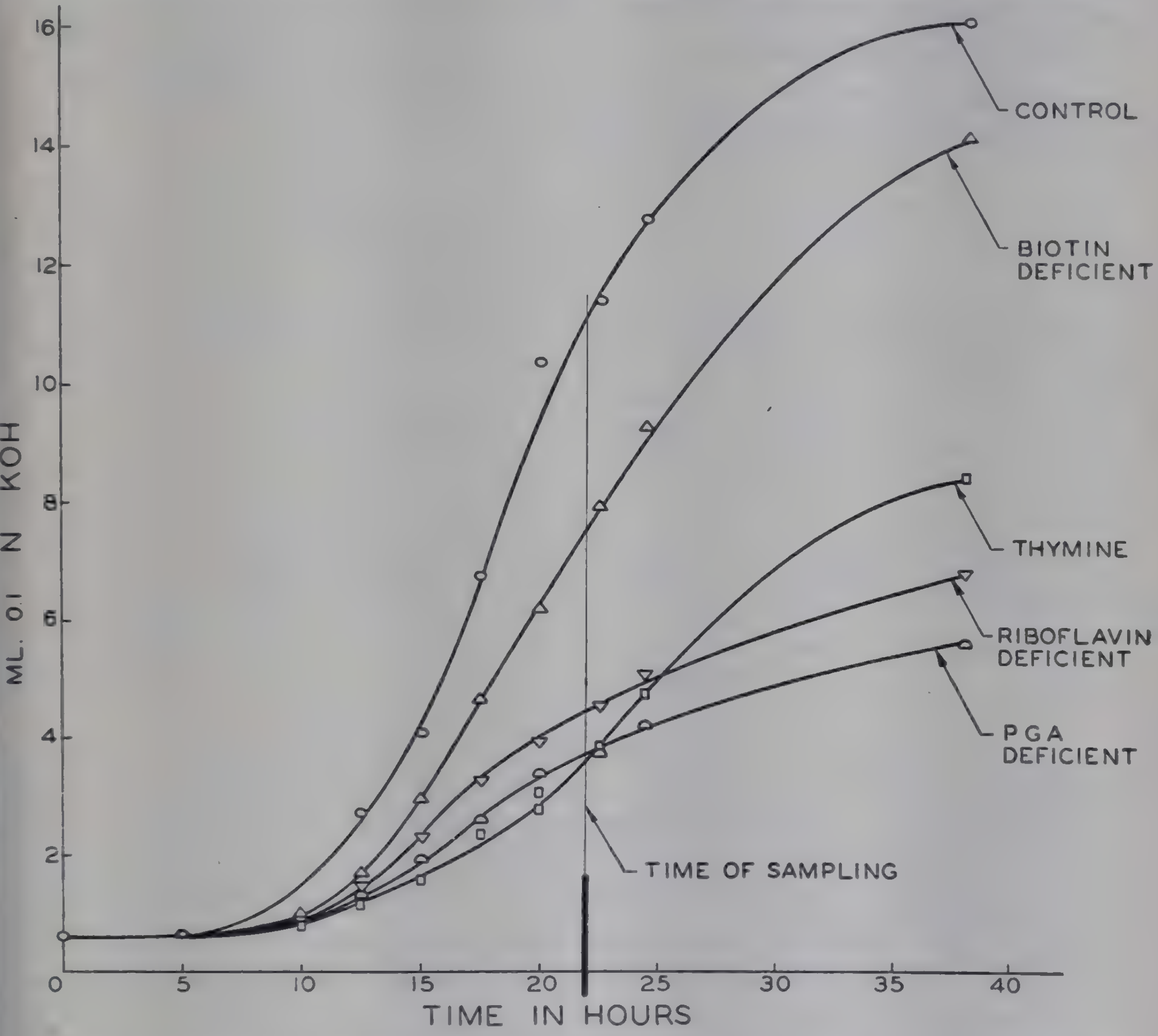


FIG. 2. Rate of acid production of *Lactobacillus casei* in media containing excess PGA (control), thymine substituted for PGA, and deficient in PGA, riboflavin, or biotin.

Results

PGA and Thymine Relationships to Nucleic Acid—A comparison was made of nucleic acid synthesis in media of varying folic acid and thymine content, bacteria in approximately the same growth phase (22 hours) being used. Data regarding growth and acid production will be noted in Figs. 1 and 2.

As shown in Table II, there was no significant difference in the ribonucleic acid content of bacterial cells when supplied with either abundant or restricted quantities of PGA. There was a significant increase in both types of nucleic acids when thymine replaced PGA in the medium. In contrast, a marked decrease in the desoxyribonucleic acid content resulted when growth was restricted by a PGA deficiency.

TABLE II

Nucleic Acids, Nitrogen, Dry Weight, and Acid Production of Lactobacillus casei Grown in Various Media[*]

Components varied in media[†]	Desoxyribonucleic acid	Ribonucleic acid	Nitrogen	0.1 N acid per 10 ml. media	Total cell mass	0.1 N acid per mg. cells
Series A, averages and ranges of five runs						
	per cent	*per cent*	*per cent*	*ml.*	*gm. per l.*	*ml.*
Control, PGA 2.0 γ per liter	1.47 1.22–1.74	12.7 11.6–13.8	8.67 8.00–9.57	10.0 8.2–11.1	1.34 1.08–1.63	0.75 0.68–0.78
PGA-deficient, 0.05 γ per liter	0.79 0.56–1.06	12.6 12.1–13.2	9.86 9.19–11.5	3.2 3.1–3.7	0.286 0.220–0.348	1.11 1.06–1.12
Thymine, 500 γ per liter	1.89 1.56–2.42	17.3 16.4–17.8	10.4 8.98–12.6	3.0 2.3–3.7	0.302 0.241–0.375	0.98 0.93–1.10
Series B, averages and ranges of four runs						
Control, PGA 2.0 γ per liter	1.42 1.33–1.51	13.6 12.9–14.7	9.09 8.00–10.2	10.9 10.8–10.9	1.42 1.41–1.42	0.77 0.76–0.78
Riboflavin-deficient, 10 γ per liter	2.08 1.97–2.35	20.0 18.1–22.9	12.1 10.6–12.8	4.1 3.9–4.2	0.212 0.202–0.226	1.93 1.82–2.08
Biotin-deficient, 0.1 γ per liter	2.42 2.31–2.50	15.8 14.5–16.8	10.9 9.20–12.0	7.0 6.4–7.6	0.518 0.363–0.690	1.38 1.00–1.76

[*] Dry weight basis after 22 hours at 37°.

[†] Standard components of the media are listed in Table I.

The quantities of acid produced per mg. of dry bacterial cells were almost identical under the conditions of PGA deficiency and when thymine replaced PGA. A higher concentration of PGA caused the acid production to be approximately tripled. Hall (6) observed a decrease in the rate of acid production as the pH of the medium fell, but the change was not marked within the pH range of the tests reported here.

A small increase in the nitrogen content of the bacterial cells was evident both as a result of growth on a PGA-deficient medium and when thymine was substituted for the vitamin.

Comparison of PGA, Riboflavin, and Biotin Deficiencies—For comparative purposes, a further study was made of nucleic acid synthesis when growth was restricted by a riboflavin or biotin deficiency. The bacteria when harvested were approaching the end of their active growth period (22 hours) in the medium deficient in biotin. In the riboflavin-restricted medium, growth practically ceased at the end of 15 hours, but acid production continued. The cells were harvested as in the preceding experiment after 22 hours of growth.

The data in Table II show that both the RNA and the DNA contents of bacterial cells subjected to biotin or riboflavin deficiency were significantly higher than in cells that were grown in a PGA-deficient medium or in a medium which contained more nearly optimum concentrations of all nutrients (control medium).

Acid productions in riboflavin- and biotin-deficient media were 38 and 64 per cent, respectively, of the quantities produced under more nearly optimum nutrient conditions. But the amounts of acid produced per mg. of dry cell mass formed in riboflavin- and biotin-deficient media were 250 and 180 per cent, respectively, of the quantities produced under optimum nutrient conditions (Table II).

The nitrogen values on a dry weight basis after growth in riboflavin- and biotin-deficient media were 133 and 120 per cent, respectively, of the control values.

DISCUSSION

The degree of PGA restriction in the medium and the time interval for culture growth were selected, in part, on the basis of producing a cell mass and an acidity that would be approximately equivalent to the respective values obtained when excess thymine replaced PGA in the medium. The contrast between desoxyribonucleic synthesis (decrease) and ribonucleic acid synthesis (no change) was sufficiently striking to point toward a specific functional rôle of the nutrient in one or more of the steps essential for synthesizing desoxyribonucleic acid.

In view of the rôle which Boiven *et al.* (23) and Belozersky (24) have suggested for desoxyribonucleic acid in bacterial cells, it would be of interest to explore further the changes that are associated with PGA deficiency. A new type of lead is provided by Shive *et al.* (25), who isolated from hog liver a crystalline compound which was several times as active as folic acid in producing one-half maximum growth and which counteracted methylfolic acid toxicity. This compound was identical with the nucleoside thymidine in x-ray diffraction pattern and biological properties. Hall has reported a product or products derived from histidine by chemical (26) and enzymatic (27) procedures with PGA activity for *Streptococcus lactis* R.

The biotin and riboflavin studies were conducted primarily to find whether the decreased desoxyribonucleic acid production might be due merely to a non-specific nutrient deficiency. No decrease in the content of the nucleic acids was observed; rather, there was a moderate increase in both cases, but no evidence of a change in proportion of the two products. Price, Miller, and Miller (28) similarly did not find a marked difference in the RNA and DNA storage in the cell nuclei of rats as a result of riboflavin deficiency.

In agreement with observations on other organisms (29, 24, 30) it was found that the nucleic acid content of *Lactobacillus casei* tended to decrease as the age of the culture increased. The RNA content, for example, was 19.5 per cent in a 15 hour culture and 14.7 per cent in the 22 hour culture. The DNA content of a 15 hour culture was 1.83 per cent, and it decreased to 1.33 per cent in the 22 hour culture. This age relationship may explain, in part, the higher nucleic acid values observed with *Lactobacillus casei* grown in riboflavin- or biotin-deficient media and in media with thymine substituted for PGA. These considerations lend emphasis to the observed effects of PGA deficiency.

The effect of thymine in increasing the nucleic acid content above that obtained in the control medium (optimum PGA) does not necessarily support Stokes' (4) hypothesis that folic acid is involved in thymine synthesis, since an increase in both types of nucleic acid also resulted from growth in a medium deficient in either riboflavin or biotin.

Among other observed influences of PGA, failure to form red or white blood cells at a normal rate is one of the most characteristic features of a deficiency in animals. Rodney, Swendseid, and Swanson (31) found that "the rate of tyrosine oxidation by livers from PGA-deficient rats was increased by the addition of PGA." Woodruff and Darby (32) reported that in the scorbutic guinea pig either ascorbic acid or PGA decreased the urinary excretion of tyrosyl derivatives and keto acids, which appeared in abnormally high concentrations due to the addition of L-tyrosine in the diet. Totter and Sims (33) reported that PGA counteracted the inhibitory effect of KCN on both growth and porphyrin production of *Corynebacterium hoffmannii*. Martin and Beiler (34) have reported that 7-methylfolic acid and the aspartic acid analogue of PGA markedly inhibited dopa decarboxylase in rat kidney preparations. In none of the above cases has there been a basis for citing the specific reactions in which PGA plays a direct rôle.

SUMMARY

A partial deficiency of pteroylglutamic acid in a medium otherwise favorable for rapid growth of *Lactobacillus casei* resulted in a distinctly lower content of desoxyribonucleic acid, while the content of ribonucleic acid was essentially unchanged.

A comparable deficiency of riboflavin or biotin did not cause a similar selective effect, but instead resulted in a moderate increase in the content of both types of nucleic acid.

An excess of thymine, adequate to afford growth and acid production rates approximately equal to the respective values resulting from pteroylglutamic acid deficiency, caused a moderate rise in both desoxyribonucleic acid and ribonucleic acid.

BIBLIOGRAPHY

1. Snell, E. E., and Mitchell, H. K., *Proc. Nat. Acad. Sc.*, **27**, 1 (1941).
2. Snell, E. E., and Peterson, W. H., *J. Bact.*, **39**, 273 (1940).
3. Stokstad, E. L. R., *J. Biol. Chem.*, **139**, 475 (1941).
4. Stokes, J. L., *J. Bact.*, **48**, 201 (1944).
5. Hitchings, G. H., Falco, E. A., and Sherwood, M. B., *Science*, **102**, 251 (1945).
6. Hall, D. A., *Biochem. J.*, **41**, 287 (1947).
7. Strandskov, F., and Wyss, O., *J. Bact.*, **52**, 575 (1946).
8. Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, **166**, 435 (1946).
9. Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, **170**, 133 (1947).
10. Rogers, L. L., and Shive, W., *J. Biol. Chem.*, **172**, 751 (1948).
11. Stokstad, E. L. R., Regan, M., Franklin, A. L., and Jukes, T. H., *Federation Proc.*, **7**, 193 (1948).
12. Stetten, M. R., and Fox, C. L., Jr., *J. Biol. Chem.*, **161**, 333 (1945).
13. Shive, W., Ackermann, W. W., Gordon, M., Getzendaner, M. E., and Eakin, R. E., *J. Am. Chem. Soc.*, **69**, 725 (1947).
14. Schopfer, W. H., *Experientia*, **2**, 188 (1946).
15. Teply, L. J., and Elvehjem, C. A., *J. Biol. Chem.*, **157**, 303 (1945).
16. Roberts, E. C., and Snell, E. E., *J. Biol. Chem.*, **163**, 499 (1946).
17. Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, **46**, 2066 (1924).
18. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).
19. Dische, Z., *Mikrochemie*, **8**, 4 (1930).
20. Levene, P. A., and Bass, L. W., Nucleic acids, American Chemical Society monograph series, New York, 298 (1931).
21. Albaum, H. G., and Umbreit, W. W., *J. Biol. Chem.*, **167**, 369 (1947).
22. Mejbaum, W., *Z. physiol. Chem.*, **258**, 117 (1939).
23. Boiven, A., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **12**, 7 (1947).
24. Belozersky, A. N., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **12**, 1 (1947).
25. Shive, W., Eakin, R. E., Harding, W. M., Ravel, J. M., and Sutherland, J. E., *J. Am. Chem. Soc.*, **70**, 2299 (1948).
26. Hall, D. A., *Biochem. J.*, **41**, 299 (1947).
27. Hall, D. A., *Biochem. J.*, **42**, p. ix (1948).
28. Price, J. M., Miller, E. C., and Miller, J. A., *J. Biol. Chem.*, **173**, 345 (1948).
29. Belozersky, A. N., *Microbiology*, **10**, 185 (1941).
30. Stokinger, H. E., Ackerman, H., and Carpenter, C. M., *J. Bact.*, **47**, 129 (1944).
31. Rodney, G., Swendseid, M. E., and Swanson, A. L., *J. Biol. Chem.*, **168**, 395 (1947).
32. Woodruff, C. W., and Darby, W. J., *J. Biol. Chem.*, **172**, 851 (1948).
33. Totter, J. R., and Sims, E. S., *Federation Proc.*, **7**, 196 (1948).
34. Martin, G. J., and Beiler, J. M., *Arch. Biochem.*, **15**, 201 (1947).

OXYBIOTIN METABOLISM IN THE CHICK*

I. DEPOSITION OF OXYBIOTIN IN TISSUES

By R. H. McCOY, JULIET N. McKIBBEN, A. E. AXELROD,
AND KLAUS HOFMANN

(*From the Department of Chemistry, University of Pittsburgh, and the Institute of Pathology, Western Pennsylvania Hospital, Pittsburgh*)

(Received for publication, August 5, 1948)

Oxybiotin, the oxygen analogue of biotin, can replace biotin in the nutrition of the rat (1), the chick (2), and certain microorganisms (3). It has been clearly established that *Saccharomyces cerevisiae* and *Rhizobium trifolii* can utilize oxybiotin directly (4, 5). Recently Krueger and Peterson (6) have obtained similar results with *Lactobacillus pentosus*. The present experiments were designed to determine whether oxybiotin is active as such or whether it is converted into biotin by the chick.

If oxybiotin is active as such, its administration to biotin-deficient animals should lead to an accumulation of oxybiotin in the tissues with no concomitant increase in their biotin content. Furthermore, there should be a correlation between the growth response of the animals and the oxybiotin content of tissues. This paper presents the results of a study of the deposition of oxybiotin and biotin in chick tissues following the intramuscular administration of these compounds. By differential assay for biotin and oxybiotin it has been possible to demonstrate that the increased "biological activity" (or biotin-like activity) of tissues (as measured microbiologically) which appears after the administration of oxybiotin to chicks is due to oxybiotin rather than biotin. The growth response of chicks was found to parallel the increase in the oxybiotin content of their tissues.

EXPERIMENTAL

Care of Animals

Day-old white Leghorn cockerels served as experimental animals throughout this study. The basal diet was essentially the same as that used previously for oxybiotin studies (2), and consisted of dextrin, 55.7 per cent; Labco "vitamin-free" casein, 20 per cent; dried raw egg white, 10 per cent; salts, 5 per cent (7); fortified corn oil,[1] 5 per cent; solubilized liver fraction

---

* This work was aided by grants from the Buhl Foundation, from the Williams-Waterman Fund of the Research Corporation, and from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council. A preliminary report of these studies has appeared (*Federation Proc.*, **6**, 276 (1947)).

[1] Per 100 gm. of fortified corn oil: α-tocopherol 160 mg., 2-methyl-1,4-naphthoquinone 20 mg., vitamin A 80,000 U. S. P. units, and vitamin D 16,000 U. S. P. units.

L,[2] 2 per cent; glycine, 2 per cent; choline chloride, 0.2 per cent; and *i*-inositol, 0.1 per cent. To each kilo of ration were added thiamine hydrochloride, 10 mg.; riboflavin, 15 mg.; pyridoxine, 15 mg.; calcium pantothenate, 50 mg.; nicotinic acid, 100 mg.; and pteroylglutamic acid,[3] 1.0 mg. After 1 week on this basal ration, the chicks were separated into four comparable groups on the basis of their weight and growth performance. One group served as a control, while the other three were injected intramuscularly on alternate days with 2, 8, or 20 γ of *dl*-oxybiotin (Table I). Two chicks from each group were sacrificed each week for the following 4 weeks, and all of the surviving chicks were sacrificed at the end of the 5th week of injections. Immediately after decapitation, the heart, liver, spleen, and a sample of leg muscle were removed from each chick and kept frozen until analyzed.

A second experiment conducted in a similar manner included the following: (*a*) a positive control group supplied with a commerical chick ration,[4] (*b*) two groups injected on alternate days with either 0.3 or 15.0 γ of *d*-biotin, (*c*) two groups injected on alternate days with either 2.0 or 30.0 γ of *dl*-oxybiotin, and (*d*) a negative control group fed the basal ration alone. After 5 weeks of injections, all chicks were sacrificed and tissues were removed for analysis (Table III).

Analytical Procedures

Preparation of Samples—Studies were made of various procedures for the extraction of biotin and oxybiotin from tissues. These included autoclaving or refluxing the minced tissue samples for various periods of time up to 6 hours with various concentrations of HCl or H_2SO_4 up to 6 N. Neither time, concentration of acid, nor choice of acid appeared to be particularly critical in these studies. Autoclaving at 15 pounds pressure for 2 hours with 30 ml. of 5 N HCl per gm. of dry weight of sample was found to liberate the largest amount of "biological activity" from tissues, and this procedure was adopted for general use. Recovery of biotin or oxybiotin added either before or after hydrolysis was good. Following the digestion, each solution was filtered, the residue washed with dilute acid and with water, and the combined filtrates evaporated to dryness. These solids were then dissolved in water, adjusted to pH 6.7 with dilute NaOH, and made up to volume. Aliquots were diluted for microbiological assay.

Microbiological Assays—All analyses were made by the Wright and Skeggs (8) microbiological procedure with *Lactobacillus arabinosus* as the test organism.[5] For this organism, 0.4 mγ of *dl*-oxybiotin is equivalent to 0.2 mγ of *d*-biotin, and the two growth curves are superimposable. The "biological activity" (or biotin-like activity) of a tissue extract, therefore, represents the response to the sum of the biotin and oxybiotin present and is expressed in terms of *d*-biotin.

---

[2] Supplied through the courtesy of Dr. David Klein, The Wilson Laboratories.

[3] Pteroylglutamic acid was kindly supplied by the Lederle Laboratories Division, American Cyanamid Company; all other water-soluble vitamins by Merck and Company.

[4] Purina chick Startena.

Two types of differential analysis for biotin and oxybiotin were used. The permanganate procedure (4), modified to use *Lactobacillus arabinosus* as the test organism, and the Raney's nickel procedure (9) were found to give comparable results. In both methods biotin is converted into a compound inactive for *Lactobacillus arabinosus*, by permanganate into the sulfone and by Raney's nickel into desthiobiotin. Any activity remaining after either of these treatments is assumed to be oxybiotin, since under the conditions of these tests oxybiotin is unaffected. Good recoveries of added oxybiotin were obtained. Thus, in a sample containing both biotin and oxybiotin, an aliquot was removed and treated by either permanganate or Raney's nickel and then assayed simultaneously with an untreated aliquot. The difference between the values of the treated and untreated aliquots was a direct measure of the biotin present in the sample.

Results

The growth response of chicks to graded doses of oxybiotin confirms the earlier observation (2) that *dl*-oxybiotin is approximately 17 per cent as active as *d*-biotin in the nutrition of the chick. Thus, chicks from the second series which received a dosage of 2.0 γ of *dl*-oxybiotin attained a greater weight (204 gm.) than those which received 0.3 γ of *d*-biotin (175 gm.), while the group receiving 30 γ of *dl*-oxybiotin weighed less (222 gm.) than those receiving 15 γ of *d*-biotin (268 gm.).

Table I summarizes the "biological activity" found in tissues from chicks injected with graded doses of oxybiotin. No significant variations were observed in the concentrations of biotin-like activity of tissues from chicks sacrificed at the varying time intervals and, therefore, all of the individual figures were included in the averages for each group. Apparently, at each dosage level, the tissue concentrations attained a constant value within 1 week after injections were started. With increasing dosages of oxybiotin a progressive increase in concentration (per gm. of fresh weight of tissue) and in total amount of biotin-like activity per organ was observed. For various tissues the concentrations of biotin-like activity found in the 20 γ dosage group were between 5 and 16 times as high as those in the control animals. The total amounts of "biological activity" found in these organs showed even larger increases because of the larger size of the individual organs in the injected animals. An examination of the relative increases in concentration of biotin-like activity with increased dosages suggests that the tissues approach saturation with a 20 γ dosage. A close parallelism was also observed between the growth response and the concentration of biotin-like activity found in individual tissues.

---

[5] L-Asparagine (0.01 gm.) was added per 100 ml. of the original single-strength medium.

The tissues from the chicks of the first series which received injections of 2 γ of *dl*-oxybiotin on alternate days for 5 weeks were subjected to differential assay for oxybiotin and biotin by the Raney's nickel procedure (9) (Table II). Tissues from chicks of the second series which received injections of 30 γ of *dl*-oxybiotin on alternate days for 5 weeks were subjected to differential assay for oxybiotin and biotin by the permanganate procedure (4). Table III summarizes these findings in comparison with the biotin content of tissues from deficient controls, chicks injected with biotin, and chicks on a commercial ration. At these two dosage levels (2 and 30 γ, respectively) the concentrations and total quantities of biotin found in tissues from chicks injected with oxybiotin and control chicks were not significantly different. However, despite the similarity of amounts of biotin found in the chicks injected with oxybiotin and control groups, the former groups averaged, respectively, 60 and 83 gm. greater increases in body weight during the 5 week period. Thus, no correlation was observed

TABLE I

"Biological Activity" of Tissues from Chicks Injected with Oxybiotin

	Controls	Oxybiotin-injected chicks		
Dosage,* γ		2	8	20
Average starting weight,† *gm.*	55	57	53	54
Average final weight,‡ *gm.*	139	201	234	254
Liver, *mγ per gm.*	420 ± 47§	810 ± 77	1,900 ± 70	2,590 ± 82
" *total mγ*	1820 (14)‖	4840 (14)	12,200 (17)	16,740 (15)
Heart, *mγ per gm.*	34 ± 5.5	96.5 ± 9.2	190 ± 10	220 ± 7.3
" *total mγ*	30 (14)	112 (14)	230 (17)	300 (15)
Spleen, *mγ per gm.*	23 ± 3.3	41 ± 4.1	85 ± 8.4	120 ± 4.6
Spleen, *total mγ.*	2.6 (13)	9 (14)	26 (17)	45 (15)
Muscle, *mγ per gm.*	12 ± 1.5 (6)	65.5 ± 12.5 (6)	120 ± 5.1 (11)	192 ± 13 (9)

* Injected on alternate days into breast muscle.

† After 1 week on basal diet.

‡ Average weight of chicks at end of experiment.

§ Mean ± standard error of the mean.

‖ The figures in parentheses are the number of tissues analyzed.

TABLE II

Biotin and Oxybiotin in Tissues from Chicks Injected with Oxybiotin

	d-Biotin		*dl*-Oxybiotin	
	total mγ	*mγ per gm.*	*total mγ*	*mγ per gm.*
Liver, injected[*]	1970 (6)[†]	300	6420 (6)	980
" control	1820 (14)	420	0	0
Heart, injected	19 (6)	13	150 (6)	110
" control	30 (14)	34	0	0
Muscle, injected	(6)	17	(6)	80
" control	(6)	12	0	0

[*] 2 γ of *dl*-oxybiotin on alternate days for 5 weeks.

[†] The figures in parentheses are the number of tissues analyzed.

TABLE III

Comparison of Biotin and Oxybiotin Content of Tissues from Various Chick Groups

Groups	Deficient controls	Oxybiotin-injected[*]		Biotin-injected[†]	Commercial diet
	d-Biotin	*d*-Biotin	*dl*-Oxybiotin	*d*-Biotin	*d*-Biotin
Liver, *mγ per gm.*	440 ± 75[‡]	356 ± 41	4,250 ± 210	3,370 ± 250	2,360 ± 60
Liver, *total mγ*	2020 (8)[§]	2240 (10)	31,530	28,000 (7)	27,000 (8)
Heart, *mγ per gm.*	38 ± 10	34 ± 3	396 ± 28	263 ± 12	274 ± 15
Heart, *total mγ*	36 (8)	36 (10)	430	356 (7)	488 (8)
Muscle, *mγ per gm.*	11 ± 1 (5)	4 ± 1 (5)	94 ± 4	61 ± 4 (5)	
Spleen, *mγ per gm.*	9 ± 1	4.3 ± 2	124 ± 5	60 ± 8	
Spleen, *total mγ*	1.6 (5)	2.5 (5)	63	28 (5)	
Lung, *mγ per gm.*		3.2 ± 1	67 ± 4	35 ± 2	52
Lung, *total mγ*		4.3 (8)	89	51 (8)	93 (3)
Starting weight, *gm.*[‖]	52	53		53	64
Final weight, *gm.*	138	222		268	412

[*] 30 γ of *dl*-oxybiotin on alternate days for 5 weeks.

[†] 15 γ of *d*-biotin on alternate days for 5 weeks.

[‡] Mean ± standard error of the mean.

[§] The figures in parentheses are the number of tissues analyzed.

[‖] After 1 week on a basal or commercial diet.

between the growth response and actual biotin content of tissues. In contrast, the growth response was found to increase with increasing tissue concentrations of oxybiotin.

When microbiologically equivalent dosages of *d*-biotin (15 γ) and *dl*-oxybiotin (30 γ) were injected into chicks, the concentrations of biotin-like activity found in chick tissues (by microbiological assay) were quite similar (Table IV). Since the increase in biological activity resulting from the injection of *dl*-oxybiotin has been shown to be due to oxybiotin, it is apparent that the storage of oxybiotin and biotin was approximately the same at these injection levels. However, the group injected with biotin on the average gained 45 gm. more than the oxybiotin group. The differences in growth performance, therefore, are not the result of differences in absorption and storage, but the direct result of intrinsic variations in the biological activities of the two molecules in the chick.

TABLE IV

"Biological Activity" of Tissues from Chicks Injected with Biotin or Oxybiotin

	Biotin-injected*	Oxybiotin-injected†
	mγ per gm.	*mγ per gm.*
Liver	3370	2480
Heart	263	232
Muscle	61	51
Spleen	60	66
Lung	35	36

* 15 γ of *d*-biotin on alternate days for 5 weeks.

† 30 γ of *dl*-oxybiotin on alternate days for 5 weeks.

TABLE V

*"Biological Activity" Liberated by Autoclaving in Acid or in Water**

		Liver	Heart	Muscle	Spleen
Autoclaved 2 hrs. in 5 N HCl	Average	2930	293	158	131
	Range	2220–3460	246–393	129–214	88–205
Autoclaved 2 hrs. in distilled H_2O	Average	172	35	35	34
	Range	98–223	22–60	24–41	17–56
% liberated in distilled H_2O	Average†	5.7	12.6	22	28
	Range	4.4–8.0	6,0–24	15–29	14–44

* Values from six chicks which received 20 γ of *dl*-oxybiotin on alternate days for 5 weeks. The values are expressed as mγ per gm. of fresh tissue.

† On assumption of 100 per cent liberation by 5 N HCl.

Since a large percentage of the biotin of animal tissues is present in a bound form, that is, requires hydrolysis with acid or enzymes for its liberation, it was of interest to determine whether the "biological activity" present in animal tissues following the injection of oxybiotin was similarly bound. Tissues from chicks which had received 20 γ of *dl*-oxybiotin on alternate days for 5 weeks were used in the experiment. Each tissue was divided into approximately equal portions and these were autoclaved for 2 hours in either 5 N HCl or in distilled water. Microbiological assays were conducted on the filtrates. The findings are summarized in Table V. It is apparent that only a small proportion of the "biological activity" was liberated by autoclaving in water. Thus, the "biological activity" stored following the injection of oxybiotin was bound in a manner comparable to that of biotin.

DISCUSSION

The storage of oxybiotin in chick tissues has been repeatedly observed during these studies. This is in marked contrast with the report by Moore, Luckey, Elvehjem, and Hart (10) that "no appreciable accumulation" occurred in either liver or muscle of the chick until 1000 γ of *dl*-O-heterobiotin (oxybiotin) was fed per 100 gm. of diet. Although no food consumption figures were reported, it is obvious that the intake of their animals was many times the 1 γ per day which in our experiments induced a large deposition of oxybiotin in muscle, heart, and liver tissue. After only 1 week of injections, sufficient oxybiotin had been deposited in liver and muscle to more than double the "biological activity," compared to deficient control tissues. Apparently the injection of small quantities of oxybiotin leads to a greater and more prompt deposition in tissues than the feeding of high concentrations in the ration. It should be noted that the differential assay employed by Moore *et al.* depends on the difference in response of *Streptococcus faecalis* R to biotin and oxybiotin. In our hands this method has not proved satisfactory.

Since biotin-deficient chicks show a definite growth response to injections of oxybiotin without any increase in the biotin content of various body tissues, and since the amount of the growth increase parallels both the amount of oxybiotin injected and the amount stored in tissues, it seems highly probable that oxybiotin is biologically active as such. The similarity of binding in tissues is in agreement with this concept. Unequivocal proof that oxybiotin is not converted into biotin in the chick is provided by complete balance experiments which are presented in the following paper.

SUMMARY

1. Liver, heart, spleen, lung, and leg muscle from chicks which received oxybiotin injections into the breast muscle were found to have a high content of oxybiotin.

2. The actual biotin content of these tissues was almost identical with that of tissues from biotin-deficient chicks.

3. The growth response of chicks injected with oxybiotin paralleled the

quantities of oxybiotin injected and the amounts of oxybiotin found in tissues.

4. When microbiologically equivalent amounts of oxybiotin and biotin were injected into similar groups of chicks, comparable amounts of both compounds were stored in chick tissues.

5. Oxybiotin, like biotin, was only partially liberated from tissues by autoclaving in distilled water, yet readily liberated by autoclaving in 5 N HCl.

These findings are in agreement with the concept that the biological activity of oxybiotin is an intrinsic property of the molecule rather than a result of its conversion into biotin.

BIBLIOGRAPHY

1. Hofmann, K., McCoy, R. H., Felton, J. R., Axelrod, A. E., and Pilgrim, F. J., *Arch Biochem.*, **7**, 393 (1945).
2. McCoy, R. H., Felton, J. R., and Hofmann, K., *Arch. Biochem.*, **9**, 141 (1946).
3. Pilgrim, F. J., Axelrod, A. E., Winnick, T., and Hofmann, K., *Science*, **102**, 35 (1945).
4. Hofmann, K., and Winnick, T., *J. Biol. Chem.*, **160**, 449 (1945).
5. Axelrod, A. E., Flinn, B. C., and Hofmann, K., *J. Biol. Chem.*, **169**, 195 (1947).
6. Krueger, K. K., and Peterson, W. H., *J. Bact.*, **55**, 693 (1948).
7. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **138**, 459 (1941).
8. Wright, L. D., and Skeggs, H. R., *Proc. Soc. Exp. Biol. and Med.*, **56**, 95 (1944).
9. Hofmann, K., Winnick, T., and Axelrod, A. E., *J. Biol. Chem.*, **169**, 191 (1947).
10. Moore, P. R., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *Proc. Soc. Exp. Biol. and Med.*, **61**, 185 (1946).

OXYBIOTIN METABOLISM IN THE CHICK*

II. OXYBIOTIN AND BIOTIN BALANCE STUDIES

BY R. H. McCOY, JULIET N. McKIBBEN, A. E. AXELROD,
AND KLAUS HOFMANN

(*From the Department of Chemistry, University of Pittsburgh, and the Institute of Pathology, Western Pennsylvania Hospital, Pittsburgh*)

(Received for publication, August 5, 1948)

Balance experiments have demonstrated clearly that oxybiotin is not converted into biotin during the growth of *Saccharomyces cerevisiae* 139, *Rhizobium trifolii* (1), or *Lactobacillus pentosus* 124-2 (2). Growth experiments with chicks, together with studies of the accumulation of oxybiotin in their tissues, suggest that no conversion occurs in the chick (3). This paper presents complete balance experiments demonstrating that the chick does not convert oxybiotin into biotin and, therefore, that the biological activity of oxybiotin is an intrinsic property of the molecule.

EXPERIMENTAL

In the first series, ten newly hatched white Leghorn cockerels of identical weights were placed in pairs in screen bottom cages over glass funnels. The excreta were washed down daily from the cages and funnels into flasks containing 2 cc. of concentrated HCl and toluene and these collections were kept in the refrigerator until analyzed. Basal diet and water were supplied *ad libitum*. Daily records of chick weights and food consumption were kept. The diet consisted of sucrose, 60.2 per cent; Labco "vitamin-free" casein, 25 per cent; salts, 5 per cent (4); fortified corn oil, 5 per cent;[1] solubilized liver fraction L, 2 per cent;[2] glycine, 2 per cent; L-cystine, 0.3 per cent; L-arginine, 0.2 per cent; choline chloride, 0.2 per cent; and *i*-inositol, 0.1 per cent. The vitamin supplement was identical with that used in previous experiments (3). After 1 week, the chicks were separated into two comparable groups. One group of six chicks served as the negative control; the other group of four was injected intramuscularly on alternate days with eight equal dosages totaling 32 γ of *dl*-oxybiotin.

---

* This work was aided by grants from the Buhl Foundation, from the Williams-Waterman Fund of the Research Corporation, and from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council. A preliminary report of these studies has appeared (*Federation Proc.*, **6**, 276 (1947)).

[1] Contained as follows per 100 gm. of fortified corn oil: α-tocopherol 160 mg., 2-methyl-1,4-naphthoquinone 20 mg., vitamin A 80,000 U. S. P. units, and vitamin D 16,000 U. S. P. units.

[2] Supplied through the courtesy of Dr. David Klein, The Wilson Laboratories.

The animals in the second series were treated in like manner except that they were placed in individual cages. This series included three equal groups (based on their weight and growth performance) of four chicks each. One group was the negative control; a second group received 10.2 γ of *d*-biotin; the third group received 68 γ of *dl*-oxybiotin. At 3 weeks of age the chicks of both series were sacrificed (chloroform) and their carcasses frozen. For analysis, these frozen carcasses were chopped into small pieces and the entire chick hydrolyzed.

For comparison, biotin determinations were made on twelve newly hatched chicks comparable with those used in the experimental groups. These were found to have a biotin content of 5.1 ± 0.3 γ per chick (mean ± standard error).

Digestions of the chicks, the excreta, and the food samples were carried out with 5 N HCl in the manner described in the previous paper (3). Differential microbiological assays for biotin and oxybiotin were made by both the permanganate (1) and the Raney's nickel methods (5), with *Lactobacillus arabinosus* as the test organism. Added biotin was completely destroyed. Good recoveries of oxybiotin were obtained.

Results

Table I summarizes the *biotin* balance studies of the individual chicks used in these experiments. The excreta samples contained approximately the same amounts of biotin for successive weeks with no increase following the injection of oxybiotin. For simplicity, only the figures for the total excretion of biotin during the 3 week period are presented. Both the permanganate and Raney's nickel procedures were used for differential analysis and both sets of figures are presented. (These analyses were performed independently in two different laboratories.) Considering the many steps involved, the results obtained with both procedures in general are in close agreement. The Raney's nickel method, however, gave somewhat higher results for the biotin content of the excreta, although added biotin was quantitatively destroyed. However, even with the Raney's nickel figures for the total biotin content of each chick plus excreta, the chicks injected with oxybiotin did not have a significantly higher content of biotin than non-injected controls (8.6 γ total for chicks injected with oxybiotin compared to 7.6 γ for non-injected controls). With the permanganate method lower quantities of biotin were found (7.1 γ). The biotin content of chicks injected with oxybiotin was identical with that of the controls and not significantly different from that of newly hatched chicks (5.8 γ in non-injected controls, 5.4 γ by permanganate and 6.0 γ by Raney's nickel in chicks injected with oxybiotin, and 5.1 γ in comparable day-old chicks).

In the first series the total amount of basal diet consumed contained from

0.37 to 0.55 γ of biotin per chick, while in the second series (supposedly identical, but made up of a different batch of ingredients) the food provided

Table I

Biotin Content of Individual Chicks and Excreta

	dl-Oxybiotin-injected animals						Control animals			*d*-Biotin-injected animals			
Dosage	Permanganate procedure			Raney's nickel procedure									
	Chick	Excreta	Total	Chick	Excreta	Total	Chick	Excreta	Total	Dosage	Chick	Excreta	Total
γ	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ
32	5.3	3.3	6.9*	5.0	3.6	6.8*	4.7	3.3	6.3*	10.2	9.8	2.9	12.7
32	5.1		6.8	5.0		6.8	4.3		6.0	10.2	11.5	2.7	14.2
32	4.3	2.2	5.4*	4.5	4.2	6.6*	5.0	2.9	6.5*	10.2	15.8	3.5	19.3
32	7.1		8.2	8.1		10.2	5.8		7.2	10.2	11.5	3.9	15.4
68	5.1	2.1	7.2	7.3	4.4	11.7	9.5	3.3	11.1*				
68	3.9	2.5	6.4	5.5	3.9	9.4	5.8		7.5				
68	7.5	1.8	9.3	7.8	2.6	10.4	5.0	1.8	6.8				
68	5.0	1.3	6.3	4.5	2.6	7.1	8.5	2.1	10.6				
							5.1	1.8	6.9				
							4.4	2.4	6.8				
Average...	5.4	1.7	7.1	6.0	2.7	8.6	5.8	1.8	7.6		12.2	3.3	15.4

* Assuming equal amounts of biotin were excreted by each chick in the pair.

Table II

Oxybiotin Content of Individual Chicks and Excreta

dl-Oxybiotin injected	Permanganate procedure				Raney's nickel procedure			
	Chick	Excreta	Total	Recovery	Chick	Excreta	Total	Recovery
γ	γ	γ	γ	*per cent*	γ	γ	γ	*per cent*
32	15.3	22.3	26.5*	83	13.8	20.3	24.0*	75
32	18.8		30.0	94	17.5		27.7	87
32	13.5	21.0	24.0*	75	10.0	16.5	18.3*	57
32	15.6		26.1	81	13.8		22.1	69
68	25.8	22.0	47.8	70	25.3	17.7	43.0	63
68	32.3	20.4	52.7	77	33.0	17.3	50.3	74
68	25.0	28.1	53.1	78	26.8	21.6	48.4	71
68	26.0	34.2	60.2	88	24.9	28.6	53.5	79

* Assuming equal amounts of oxybiotin were excreted by each chick in the pair.

from 1.2 to 2.0 γ. The excretion by the non-injected control chicks of amounts of biotin greater than that present in the food consumed suggests that bacterial synthesis of small amounts of biotin may have occurred.

Analysis of comparable chicks which received biotin injections indicated that a large amount of the biotin could be recovered in the chick plus excreta (see Table I). Thus, if appreciable quantities of biotin were formed from oxybiotin in chicks injected with oxybiotin, it should have been detectable. The failure to find any increase in the total biotin of the chick or excreta following the injection of large dosages of oxybiotin excludes the possibility of oxybiotin being converted into biotin by the chick.

In Table II are presented the recoveries of oxybiotin injected into chicks. With a dosage of 32 γ of *dl*-oxybiotin 31 to 59 per cent was recovered within the chick, while 30 to 35 per cent was recovered in the excreta. 75 per cent or more of the injected oxybiotin was accounted for in the chick plus excreta. With the higher dosage of 68 γ of oxybiotin, 37 to 48 per cent was recovered within the chick and 25 to 50 per cent in the excreta. Certainly the recovery of oxybiotin was very good under the conditions of these experiments. The high recovery of oxybiotin precludes the possibility of its being converted into biotin.

DISCUSSION

The biotin balance experiments described in this paper demonstrate clearly that the chick does not convert oxybiotin into biotin. This conclusion is further supported by the fact that about 75 per cent of the injected oxybiotin could be accounted for unchanged in the chick plus excreta. Recovery of injected biotin was of the same order.

It must be concluded that the increased growth consistently observed following the administration of oxybiotin to biotin-deficient chicks is the result of the direct utilization of this compound. The experiments reported here complete the evidence for the first demonstration that the sulfur atom of biotin is not essential for the biological activity of this compound in a higher animal. Since several widely different species can utilize oxybiotin as such, it seems reasonable to predict that oxybiotin can replace biotin in all biological forms.

SUMMARY

1. The biotin content of chicks injected with oxybiotin was identical with that of control chicks and not significantly different from that of newly hatched chicks.

2. The biotin excretion by chicks injected with oxybiotin was identical with that of control animals.

3. When total dosages of 32 or 68 γ of *dl*-oxybiotin were injected into chicks over a 2 week period, approximately 75 per cent of the oxybiotin was accounted for almost equally distributed between the chick and excreta. A similar recovery of injected biotin was observed.

4. Since the chick does not convert oxybiotin into biotin, the biological activity of this compound must be an inherent property of the molecule.

BIBLIOGRAPHY

1. Hofmann, K., and Winnick, T., *J. Biol. Chem.*, **160**, 449 (1945).
2. Krueger, K. K., and Peterson, W. H., *J. Bact.*, **55**, 693 (1948).
3. McCoy, R. H., McKibben, J. N., Axelrod, A. E., and Hofmann, K., *J. Biol. Chem.*, **176**, 1319 (1948).
4. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **138**, 459 (1941).
5. Hofmann, K., Winnick, T., and Axelrod, A. E., *J. Biol. Chem.*, **169**, 191 (1947).

SYNTHESIS OF CARBONYL-LABELED PYRUVIC ACID*

By H. S. ANKER

(*From the Department of Biochemistry, University of Chicago, Chicago*)

(Received for publication, July 12, 1948)

Carbonyl-labeled pyruvic acid was synthesized from radioactive carbon dioxide for use in feeding experiments, the results of which are reported in the following communication (1).

The synthesis involves the following steps.

$$\overset{*}{C}O_2 \rightarrow CH_3\overset{*}{C}OOK \rightarrow CH_3\overset{*}{C}OBr \rightarrow CH_3\overset{*}{C}OCN \rightarrow CH_3\overset{*}{C}OCONH_2 \rightarrow CH_3\overset{*}{C}OCOOH$$

1-C^{14} Potassium Acetate—The method described here does not differ in principle from those published earlier (2). The apparatus used is illustrated in Fig. 1. 25 mM of radioactive barium carbonate are introduced into the generator, 50 ml. of hydrochloric acid-water, 1:10, into the dropping funnel, and a saturated barium hydroxide solution into the last trap. After a moderate stream of nitrogen has been started, 100 ml. of an ethereal 0.5 N methyl magnesium bromide solution[1] are pipetted into the reaction vessel, which is cooled in an ice bath. After all the air has been displaced, 100 ml. of 0.1 N sodium hydroxide solution are added to the gas absorption bottle.[2] The nitrogen stream is reduced and the hydrochloric acid added rapidly to the barium carbonate. The reaction vessel is continuously shaken. When the barium carbonate is decomposed, the nitrogen stream is increased again. The reaction product precipitates as the carbon dioxide enters the reaction vessel. After shaking for 20 to 30 minutes, 50 ml. of ice water are introduced into the reaction vessel. When the precipitate has dissolved, 30 ml. of 2 N sulfuric acid are added and the ice bath removed. All additions are made after pressure equalization and without interruption of the nitrogen stream, which is continued until the ether layer has evaporated completely. 60 mM of silver sulfate and 50 ml. of concentrated sulfuric acid are added and the mixture is distilled with steam in an all-glass distilling apparatus. The distillate is neutralized with 0.1 N potassium hydroxide and concentrated to dryness. The residue is dissolved in 100 ml. hot methanol and filtered after addition of a small quantity of charcoal. The filtrate is again evaporated and dried. The yield is 65 to 85 per cent, based on the barium carbonate used. Nearly all unchanged carbon dioxide can be recovered from the alkali trap. (Only a very small quantity of barium carbonate should have precipitated in the barium hydroxide trap.)

---

* This work was supported in part by a grant from the United States Public Health Service.

[1] Arapahoe Chemicals, Boulder, Colorado.

[2] Barium hydroxide solution is unsuitable here, as precipitated barium carbonate may clog the fritted disk.

1-C^{14} Acetyl Bromide (*3*)—20 mM of potassium acetate are pulverized, mixed with 16 mM of benzoic acid, and added to a distilling flask. Two portions of 2 mM each of benzoic acid are used to "wash" out the flask which contained the potassium acetate. 10 ml. of benzoyl bromide are added to the mixture, a glass wool plug is inserted below the side-arm, and the flask heated carefully so that the acetyl bromide distils slowly into the tared receiving flask. The boiling range is 72–76°. Yield, 75 to 90 per cent.

1-C^{14} Acetyl Cyanide (*4*)—8 mM of acetyl bromide are added to 9 mM of dry cuprous cyanide contained in an ice-cooled ampul. Sufficient dry cyclohexane to wet all the cuprous cyanide is added and the ampul is sealed off. After standing for 3 days at room temperature the contents are extracted with five portions of 2.5 ml. each of dry ether. The ether solution is used directly. Yield, 75 to 80 per cent.

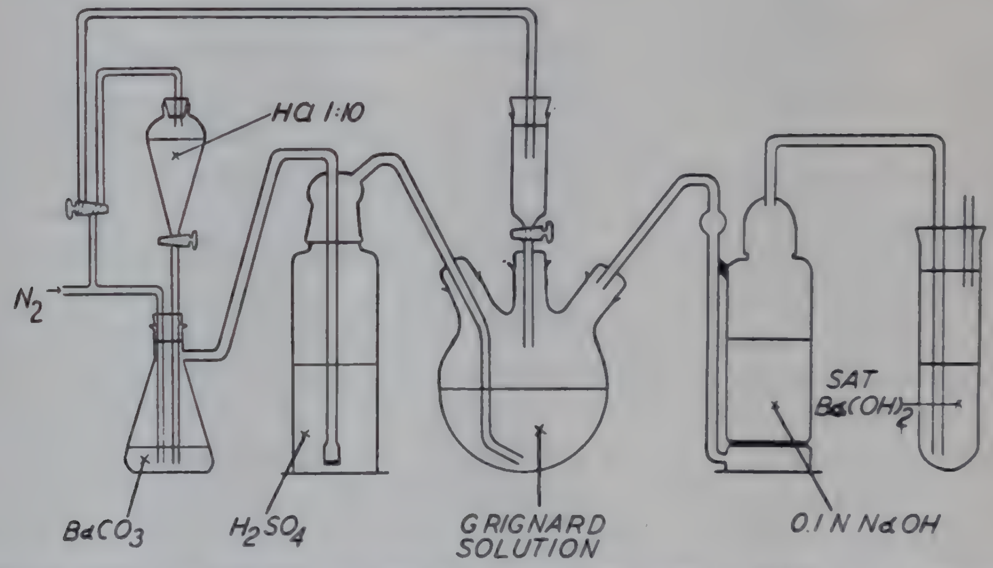


FIG. 1. Apparatus for the synthesis of labeled acetic acid. (The wash bottles are Corning catalogue, Nos. 31770 and 31750.)

2-C^{14} Pyruvamide (*5*)—The ether solution is introduced into a jacketed funnel with a fritted filter disk through which dry hydrogen chloride gas passes from the bottom. Ice water is circulated through the jacket and a calcium chloride tube is attached at the top. After the solution is saturated with hydrogen chloride, 6.5 mM of water are added to the ether solution and the hydrogen chloride stream continued for 30 minutes. Pyruvamide crystallizes out. The hydrogen chloride stream is discontinued and the material filtered by gravity. The precipitate is washed twice with 5 ml. each of ether-cyclohexane, 5:1, saturated with hydrogen chloride gas. The top of the funnel is immediately connected to a nitrogen stream in order to drive off the hydrogen chloride completely (about 3 hours). The pyruvamide obtained is recrystallized from a minimum amount of dry ethyl acetate. Yield, 40 to 70 per cent; m.p. 127°.

2-C^{14} Pyruvic Acid—A solution of pyruvic acid is obtained by dissolving 1 mM of pyruvamide in 1 to 3 ml. of water, adding 1 ml. of 1 N hydrochloric acid, and heating on a steam bath for 90 minutes. The pyruvic acid content is determined colorimetrically (6). In view of the known instability of pyruvic acid, the solution should be used as soon as possible. Yield, 90 to 100 per cent.

SUMMARY

Pyruvic acid labeled with isotopic carbon in the carbonyl group was synthesized from isotopic carbon dioxide as starting material in about 40 per cent over-all yield. Potassium acetate, acetyl bromide, acetyl cyanide, and pyruvamide were obtained as intermediates in the synthesis.

The product was stored in the form of pyruvamide rather than pyruvic acid in view of the stability of this compound.

BIBLIOGRAPHY

1. Anker, H. S., *J. Biol. Chem.*, **176**, 1337 (1948).
2. Ruben, S., Allen, M. B., and Nahinsky, P., *J. Am. Chem. Soc.*, **64**, 3050 (1942).
3. German patent 123,052; *Chem. Zentr.*, **2**, 518 (1901).
4. Tischelinzeff, W., and Schmidt, W., *Ber. chem. Ges.*, **62**, 2210 (1929).
5. Claisen, L., and Shadwell, J., *Ber. chem. Ges.*, **11**, 1566 (1901).
6. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943).

SOME ASPECTS OF THE METABOLISM OF PYRUVIC ACID IN THE INTACT ANIMAL*

By H. S. ANKER

(From the Department of Biochemistry, University of Chicago, Chicago)

(Received for publication, July 12, 1948)

Until the advent of the isotope technique, the formation of fat in adult animals was held to occur only on a diet containing an excess of calories. From the work of Schoenheimer and Rittenberg (1) it became clear that body fat is not an inert energy store but participates actively in intermediary metabolism. Their evidence also indicated that small molecules were utilized to build up the fatty acid chains as well as the cholesterol structure. The observation that carbohydrate can serve as a precursor for body fat is very old, and many investigations have been carried out to analyze the details of this process. It is clear that on a balance basis fat must ultimately be derived from the carbohydrate or protein of the diet if no fat is supplied in the food. Stetten and Boxer (2) concluded from experiments with heavy water that a large portion of dietary carbohydrate is metabolized by way of fat. These authors (3) proposed too that interference with fatty acid synthesis is one of the main metabolic defects in alloxan diabetes. Investigations by Bloch and Rittenberg (4, 5) revealed the importance of acetic acid as a precursor in the synthesis of fatty acids and cholesterol and established the quantitative significance of acetic acid as a major intermediate of metabolism.

Smedley and Lubrzynska (6) first suggested pyruvic acid as an important intermediate in carbohydrate metabolism, and many investigations since have borne out the central rôle of pyruvic acid in glycolysis. It seems likely that pyruvic acid is also a key intermediate in the conversion of carbohydrate to other body constituents. The work presented here was undertaken to ascertain directly the utilization of pyruvic acid for the various metabolic processes, particularly the synthesis of acetic acid, fatty acids, and cholesterol. For this purpose pyruvic acid labeled in the the carbonyl carbon with C^{14} was synthesized and fed to rats. Feeding experiments were also carried out with carboxyl-labeled pyruvic acid and labeled acetic acid.

EXPERIMENTAL

Syntheses of 1-C^{14} Acetic Acid and 2-C^{14} Pyruvic Acid—These preparations are described in the preceding communication (7).

---

* This work was supported by a grant from the United States Public Health Service.

$$CC = \frac{RIC}{Q + (1 - Q)RIC}$$

where Q is the quantity of the compound fed in mM per 100 gm. of rat tissue per day.

The "concentration coefficient" affords the "relative isotope concentration" which would have been found if exactly 1 mM per 100 gm. per day had been fed.

In cases in which the administered compounds contain several carbon atoms of which only one is labeled, the concentration coefficient may not be a true measure of the utilization of the test substance. The possibility exists that not all carbon atoms of the fed material are incorporated to an equal extent into the isolated compounds. In such a case the "specific concentration coefficient"[2] (*SCC*) relating only to the carbon atoms utilized will be of greater significance.

$$SCC = CC \times f$$

where f is the fraction of carbon atoms of fed compound utilized.

In Tables I, II, and V the "specific concentration coefficient" is given only in those instances in which the mechanism could be established experimentally, such as the utilization of carbon atoms 2 and 3 of pyruvic acid only for the acetyl groups and the fatty acids.

Results

The analytical data are given in Tables I to VI. The isotope concentrations found indicate that pyruvic acid is incorporated to a varying degree into the acetyl groups of the excreted foreign amines, liver glycogen, cholesterol, and fatty acids.

In order to evaluate the extent to which a labeled precursor is utilized for the synthesis of a body constituent, it is necessary to know the isotope concentration of the precursor at the site of synthesis. This value depends on the dilution of the administered precursor by its endogenous analogue, *i.e.* the size of the metabolic pool. In the case of pyruvic acid it was not possible to determine the size of the pool directly. However, an indirect estimate was obtained in view of the fact that pyruvic acid provides acetyl groups for the acetylation of some foreign amines.

Acetyl Groups—It is possible to determine the isotope concentration of the endogenous acetyl pool of the liver by taking advantage of the acetyla-

---

defined here are similar to the "coefficients of utilization" of Bloch and Rittenberg (17). The "coefficients of utilization" were used to describe the efficiency of several test substances in forming the same metabolite, while the "concentration coefficients" are used to indicate the efficiency of a precursor for the formation of several different metabolites.

tion reaction. The utilization of a labeled compound as a source of acetyl can therefore be determined by an isotope analysis of the acetyl derivative excreted in the urine. Bloch and Rittenberg (9) had shown that acetic acid itself acetylates *p*-aminobenzoic acid as well as γ-phenylaminobutyric acid, *i.e.* both aromatic and aliphatic foreign amines. They found on the other hand that alanine, presumably by way of pyruvic acid, is capable of providing the acetyl group for the acetylation of γ-phenylaminobutyric acid only, but not for *p*-aminobenzoic acid.

TABLE I

Isotope Concentrations of Urinary Excretion Products after Feeding Labeled Pyruvic and Acetic Acids

Experiment No.	Compound fed			Acetyl groups of acetyl-					
				p-Aminobenzoic acid			γ-Phenylaminobutyric acid		
		Per 100 gm. per day	Isotope concentration*	*RIC*†	*CC*†	*SCC*†	*RIC*	*CC*	*SCC*
		mM		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1‡	2-C^{14} pyruvic acid	0.46	8,400	0.14	0.32	0.21	2.0	4.3	2.9
2‡	1-C^{14} acetic "	0.45	12,600	3.5	7.6	7.6	2.7	5.9	5.9
3§	2-C^{14} pyruvic "	0.50	12,300	1.0	2.0	1.3	1.1	2.0	1.3
3§	1-C^{13} acetic "	0.47	11.9				2.1	4.3	4.3
4§	2-C^{14} pyruvic "	0.50	12,300	1.2	2.4	1.6	0.77	1.5	1.0
4§	1-C^{13} acetic "	1.04	11.9	4.6	4.4	4.4	4.9	4.7	4.7
5§	1-C^{13} pyruvic "	1.07	17.0	0.19	0.18	0.18			

* Counts per minute of $BaC^{14}O_3$, or atom per cent excess C^{13}.

† *RIC* = relative isotope concentration, *CC* = concentration coefficient, and *SCC* = specific concentration coefficient.

‡ Rats of the laboratory strain.

§ Rats of Sprague-Dawley strain.

Similar results were obtained after feeding labeled pyruvic acid to rats of the laboratory strain. The analytical values (Table I) indicate in particular that there is no significant conversion of pyruvic acid to acetic acid, as in this strain the isotope concentration of acetyl-*p*-aminobenzoic is very low. From the experiment with carboxyl-labeled pyruvic acid it is clear that only a negligible fraction of the carboxyl carbon atoms of pyruvic acid is incorporated into the acetyl groups. Only carbon atoms 2 and 3 of pyruvic acid are therefore utilized in the acetylation reaction. The use of the "specific concentration coefficients" for these data is thus justified, the factor *f* being equal to 0.67.

In the Sprague-Dawley strain, the acetyl groups of γ-phenylaminobutyric acid and *p*-aminobenzoic acid have nearly equal isotope concentrations, indicating a considerable difference of pyruvic acid metabolism in the two strains. This result would seem to indicate that a portion of the fed pyruvic acid is decarboxylated to acetic acid, which in turn provides the acetyl groups for both foreign amines. The possibility cannot be excluded that Sprague-Dawley rats are capable of using pyruvic acid directly, *i.e.* without conversion to acetic acid, for the acetylation of *p*-aminobenzoic acid. However, this explanation seems less plausible. If large quantities of labeled acetic acid are formed from the ingested pyruvic acid, it becomes impossible to evaluate the direct incorporation of carbon atoms of pyruvic acid into other compounds, such as fatty acids and cholesterol, for which acetic acid too is a precursor (3).

Pyruvic Acid Pool—A calculation of the hepatic pyruvic acid pool and its isotope concentration can be attempted from the data obtained in the laboratory strain, since after feeding pyruvic acid, the isotope content of the acetyl group of acetyl-*p*-aminobenzoic acid is very small, and therefore no appreciable conversion of pyruvic acid to acetic acid could have taken place. Therefore the appearance of the label in the acetyl group of acetyl-γ-phenylaminobutyric acid will be the result of direct acetylation by pyruvic acid; *i.e.*, condensation of pyruvic acid with the amine and subsequent decarboxylation of the condensation product (18). The isotope concentration of this acetyl group depends on the isotope concentration of the pyruvic acid in the metabolic pool and on the relative proportion of acetyl groups of γ-phenylaminobutyric acid derived from pyruvic and acetic acids respectively.

It can be seen (Table I) that after feeding labeled acetic acid the relative isotope concentrations of the acetyl groups of acetyl-*p*-aminobenzoic acid and acetyl-γ-phenylaminobutyric acid in the laboratory strain are not identical. The acetyl group of acetyl-*p*-aminobenzoic acid is derived from acetic acid only and thus provides a measure of the isotope concentration of the acetic acid pool, while the isotopic concentration of the acetyl group of acetyl-γ-phenylaminobutyric acid is lower. If it is assumed that this difference is due to direct acetylation by pyruvic acid, then the relative contribution of acetic and pyruvic acids for the acetylation of phenylaminobutyric acid can be calculated.

$$R = \frac{I'_a}{I_a - I'_a}$$

where R is the ratio of acetylation of γ-phenylaminobutyric acid by acetic to that by pyruvic acid, I_a is the isotope concentration of the acetyl group of acetyl-*p*-aminobenzoic acid, and I'_a is the isotope concentration of the acetyl group of acetylphenylaminobutyric acid.

From the data of Experiment 2 (Table I) R is found equal to 3.5, *i.e.*, acetic acid acetylates γ-phenylaminobutyric 3.5 times as fast as does pyruvic acid. From this ratio and from the isotope concentration of the acetyl group of acetyl-γ-phenylaminobutyric acid after feeding labeled pyruvic acid, the isotope concentration of the pyruvic acid actually employed for acetylation and therefore the isotope concentration in the metabolic pool can be calculated.

$$I_p = I'_p(R + 1)$$

where I_p is the isotope concentration of the pyruvic acid pool, and I'_p is the isotope concentration of the acetyl group of phenylaminobutyric acid. The "relative isotope concentration" of the pyruvic acid in the pool is calculated to be 13 per cent.

The size of the pyruvic acid pool is then given by

$$P_p = \frac{(i_p - I_p)}{I_p} F_p$$

where P_p is the pyruvic acid pool in millimoles per 100 gm. of rat tissue per day, i_p is the isotope concentration of administered pyruvic acid, and F_p is the quantity of administered pyruvic acid in millimoles per day.

If this calculation is carried out, it is found that the pyruvic acid pool is about 6 to 7 mM per 100 gm. per day. Since this calculation depends on several isotope determinations in different animals, it can be considered indicative of an order of magnitude only.

In comparison the acetic acid pool is found to be about 12 to 15 mM per 100 gm. per day in the laboratory strain and about 20 to 25 mM per 100 gm. per day in the Sprague-Dawley strain. These values are in good agreement with the results obtained by Bloch and Rittenberg (9).

The size of the pyruvic acid pool is far smaller than the quantity of carbohydrate in the diet. It is comparable to the amount of pyruvic acid which could arise from a quantity of antiketogenic amino acid equivalent to that contained in the dietary protein. It would appear, therefore, that in the liver only a small part of the administered carbohydrate is in equilibrium with pyruvic acid in the laboratory strain.

For the Sprague-Dawley strain a similar calculation of the pyruvic acid pool cannot be made, since pyruvic acid provides labeled acetyl groups also for *p*-aminobenzoic acid. Therefore it is not possible to determine the relative contributions of pyruvic and acetic acids to the isotope content of the acetyl groups of γ-phenylaminobutyric acid.

Liver Glycogen—The incorporation of isotopic carbon from labeled pyruvic acid into liver glycogen is quite variable in different animals but lies within the same range in both rat strains. The half life time of liver glycogen was determined by Stetten and Boxer (2) to be about 1 day. On the basis of this value about 85 per cent of the liver glycogen should have been replaced by newly formed glycogen in the 3 day feeding period. From the data in Table II it can be estimated that in the laboratory strain the isotope concentration of the liver glycogen is only about one-thirtieth the isotope concentration of the pyruvic acid pool. A major part of the liver glycogen must therefore have been derived from unlabeled sources, presumably from dietary carbohydrate directly. This observation is in agreement with the results obtained by Vennesland *et al.* (19), who observed that the extra glycogen deposited after administration of labeled lactic acid to fasted rats contained only a small fraction of the label.

The isotope from carbonyl- as well as from carboxyl-labeled pyruvic acid is incorporated into liver glycogen to an equal extent, indicating the utilization of all carbon atoms of pyruvic acid for glycogen formation.

Table II

Isotope Concentrations of Liver Glycogen and Urea after Feeding Labeled Pyruvic and Acetic Acids

The results are expressed in per cent.

Experiment No.	Compound fed	Liver glycogen		Urea		
		RIC	*CC*	*RIC*	*CC*	*SCC*
1*	2-C^{14} pyruvic acid	0.17	0.38			
2*	1-C^{14} acetic "	0.03	0.06			
3†	2-C^{14} pyruvic "	0.26	0.51	0.43	0.84	0.28
4†	2-C^{14} " "	0.16	0.32	0.41	0.83	0.28
4†	1-C^{13} acetic "	0.13	0.12	0.50	0.49	0.25
5†	1-C^{13} pyruvic "	0.48	0.41	0.85	0.80	0.27

* Rats of the laboratory strain.

† Rats of Sprague-Dawley strain.

Acetic acid carbon is incorporated to a small extent only into the liver glycogen.

Urea—From experiments by Mackenzie and du Vigneaud (20) it is known that the urea carbon and the respiratory carbon dioxide have the same isotope concentrations. The "specific concentration coefficients" were calculated after the administration of 2-C^{14} pyruvic acid, 1-C^{13} pyruvic acid, and 1-C^{13} acetic acid to rats of the Sprague-Dawley strain, with $f = \frac{1}{3}$ for the pyruvic acids and $f = \frac{1}{2}$ for acetic acid. These data are given in Table II and suggest an analogous rate of metabolism for pyruvic and acetic acids in these animals.

Glycine—Hippuric acid was isolated from the urine of rats which had been fed benzoic acid together with carbonyl- or carboxyl-labeled pyruvic acid or carboxyl-labeled acetic acid. The "relative isotope concentrations" given in Table III indicate that acetic acid is not a precursor for glycine.

The isotope concentration of labeled glycine was of the same order of magnitude after feeding either carbonyl- or carboxyl-labeled pyruvic acids. Degradation by ninhydrin (21) of the glycine obtained after feeding carbonyl-labeled pyruvic acid indicates that over 80 per cent of the isotope was present in carbon atom 2. It is therefore concluded that carbon atoms 1 and 2 of pyruvic acid are the precursors of carbon atoms 1 and 2 of glycine respectively. In view of the fact that Shemin (10) has demonstrated the conversion of serine to glycine, it seems most reasonable to assume that the utilization of carbon atoms 1 and 2 of pyruvic acid for glycine synthesis proceeds via serine as an intermediate. Chargaff and Sprinson (22) have proposed a mechanism for the conversion of serine to pyruvic acid. If this reaction series were reversible, it could account for the results obtained in this experiment. A similar observation was made by Buchanan *et al.* (23) who observed the conversion of lactic acid to glycine in the pigeon. Comparing the isotope concentrations of the different carbon atoms of uric acid after labeled glycine feeding with labeled lactic acid administration, the authors reached the conclusion that carbon atoms 1 and 2 of lactic acid are utilized for glycine formation in the pigeon.

TABLE III

Incorporation of Isotopic Carbon into Glycine from Labeled Acetic and Pyruvic Acids

Experiment No.	Compound fed			Glycine from hippuric acid	
		Per 100 gm. per day	Isotope concentration*	*RIC*	*CC*
		mM		*per cent*	*per cent*
5†	1-C^{14} acetic acid	0.22	87,000	0.008	0.04
5†	1-C^{13} pyruvic "	1.1	17.0	0.34	0.25
6†	2-C^{14} " "	0.5	12,300	0.35	0.70

* Counts per minute of $BaC^{14}O_3$ or atom per cent excess C^{13}.

† Rats of Sprague-Dawley strain.

Cholesterol—Experiments by Bloch and Rittenberg (4) have demonstrated the importance of acetic acid as a precursor of cholesterol. The contribution of acetic acid carbon atoms for the formation of liver cholesterol in the present experiments can be estimated from the turnover rate and the concentration of the acetic acid pool. It can be assumed that about 30 per cent of the liver cholesterol was newly synthesized in the 3 day feeding period, based on a half life time of 5 to 6 days (5, 9). The fraction derived from the carboxyl carbon atom of acetic acid is calculated to be 27 to 33 per cent of all carbon atoms of cholesterol in both rat strains. This value is in good agreement with the results obtained by Bloch and

Rittenberg (4) who found acetic acid to provide over 50 per cent of the carbon atoms of cholesterol. Experiments by these authors (5) indicate a larger utilization of the methyl than of the carboxyl carbon atom of acetic acid.

By a similar calculation it can be estimated that in the laboratory strain of rats about 7 to 8 per cent of the cholesterol carbon is derived from the carbonyl carbon atom of pyruvic acid. In this instance direct utilization of pyruvic acid is probable because, as has been shown, no conversion of pyruvic to acetic acid occurs in the laboratory strain. The extent of incorporation of pyruvic acid carbon atoms cannot be calculated in the Sprague-Dawley strain, as decarboxylation of pyruvic to acetic acid does occur for reasons mentioned above.

TABLE IV

Isotope Concentrations of Liver and Carcass Cholesterol after Feeding Labeled Pyruvic and Acetic Acids

The results are expressed in per cent.

Experiment No.	Compound fed	Liver cholesterol		Carcass cholesterol	
		RIC	*CC*	*RIC*	*CC*
1*	2-C^{14} pyruvic acid	0.39	0.85	0.10	0.23
2*	1-C^{14} acetic “	0.66	1.5	0.10	0.23
3†	2-C^{14} pyruvic “	0.16	0.31	0.14	0.28
4†	2-C^{14} “ “	0.09	0.18	0.08	0.15
4†	1-C^{13} acetic “	0.77	0.73	0.12	0.11
5†	1-C^{13} pyruvic “	0.14	0.13		

* Rats of the laboratory strain.

† Rats of Sprague-Dawley strain.

From experiments in the Sprague-Dawley strain with carboxyl-labeled pyruvic acid, it appears probable that pyruvic acid as a whole is utilized to some extent for cholesterol synthesis. After feeding carboxyl-labeled pyruvic acid the "relative isotope concentration" of liver cholesterol was found to be about half of that which is obtained after administration of carbonyl-labeled pyruvic acid. The low analytical value does not permit a quantitative evaluation of this finding but suggests at least a partial utilization of the carboxyl carbon atom of pyruvic acid.

In view of the uncertainty of the contributions of the methyl carbon atom of acetic acid and of the methyl and carboxyl carbon atoms of pyruvic acid in these experiments, the "concentration coefficients" only are reported in Table IV. It is therefore not possible to set up a complete carbon balance from the data. Other precursors can be involved to a small extent only in the synthesis of cholesterol.

The "relative isotope concentrations" of the carcass cholesterols are found to be only slightly lower than those of the liver cholesterols. Since in adult animals the isotope concentration of liver cholesterol is found to be several times the isotope content of carcass cholesterol, the liver has been suggested as the site of cholesterol synthesis (9). The similarity of the values found in the experiments reported here is most probably due to the use of growing animals. In an unreported experiment carried out with an adult rat under comparable conditions, the isotope concentration of the carcass cholesterol is only one-third of the liver cholesterol.

Fatty Acids—Rittenberg and Bloch (5) have found that after feeding carboxyl-labeled acetic acid only the odd carbon atoms of the isolated fatty acids contained the label. They concluded that their finding made it mandatory to assume that fatty acids are synthesized from C_2 units, that acetic acid is a precursor for C_2 units, and that the C_2 units from acetic acid are utilized at random for the entire fatty acid chain. This concept necessarily requires formation of C_2 units from all other fatty acid precursors and random utilization of these C_2 units. Rittenberg and Bloch considered that the C_2 units from acetic acid are not necessarily identical to the C_2 units from other precursors and suggested the formation of acylpyruvic acids as intermediates with subsequent decarboxylation of the condensation products.

TABLE V

Isotope Concentrations of Liver and Carcass Fatty Acids after Feeding Labeled Pyruvic and Acetic Acids

The results are expressed in per cent.

Experiment No.	Compound fed	Liver fatty acids						Carcass fatty acids					
		Saturated			Unsaturated			Total			Saturated		
		RIC	*CC*	*SCC*	*RIC*	*CC*	*SCC*	*RIC*	*CC*	*SCC*	*RIC*	*CC*	*SCC*
1*	2-C^{14} pyruvic acid	1.1	2.3	1.5	0.45	0.99	0.66	0.11	0.25	0.17	0.16	0.34	0.22
2*	1-C^{14} acetic "	0.71	1.6	1.6	0.22	0.49	0.49	0.06	0.13	0.13	0.05	0.11	0.11
3†	2-C^{14} pyruvic "	0.42	0.83	0.56	0.15	0.30	0.20	0.16	0.32	0.21	0.21	0.42	0.28
4†	2-C^{14} " "	0.41	0.81	0.54	0.20	0.39	0.26	0.15	0.30	0.20	0.20	0.40	0.27
4†	1-C^{13} acetic "	1.7	1.6	1.6	0.42	0.40	0.40	0.29	0.29	0.29	0.52	0.50	0.50
5†	1-C^{13} pyruvic "	0.0											

* Rats of the laboratory strain.

† Rats of Sprague-Dawley strain.

The experiments presented here are in full agreement with the hypothesis of Rittenberg and Bloch. The data are given in Table V. After feeding carboxyl-labeled pyruvic acid the isotope concentration of the liver fatty acids was found to be negligible. From this result it can be concluded that only carbon atoms 2 and 3 of pyruvic acid are used in fatty acid synthesis. Decarboxylations of saturated fatty acids isolated after feeding carbonyl-labeled pyruvic acid yielded carbon dioxide, the isotope concentration of which was about twice that of the fatty acids. This indicates that the carbonyl carbon atom of pyruvic acid is incorporated in a random manner into the odd numbered positions of the fatty acid chain (Table VI). Accordingly, in Table V the "specific concentration coefficients" are calculated with $f = 0.67$.

TABLE VI

Distribution of Isotopic Carbon in Fatty Acid Chains after Administration of Labeled Pyruvic Acid

Experiment No.	Isotope concentration		
		Found	Calculated (b)
		counts per min.	*counts per min.*
7*	Saturated fatty acids, carcass	121(a)	
	Carbon dioxide, decarboxylation	225	241†
	Ketone, decarboxylation	119	118‡
1	Saturated fatty acids, liver	87(a)	
	Carbon dioxide, decarboxylation	156	174†
	Ketone, decarboxylation	90	84‡

* In this experiment pyruvic acid was injected.

† Calculated from $b = 2a$.

‡ Calculated from $b = (32/33)a$, assuming the average chain length of the fatty acids to be 17 carbon atoms.

In the laboratory strain of rats, in which it is possible to calculate the isotope concentration of the pyruvic and acetic acid pools, an estimate of the extent of each compound as a precursor for the fatty acids of liver can be made. With a value of 1.9 days for the half life time of the liver fatty acids (4), about two-thirds of the maximum isotope concentration will have been reached in the 3 day feeding period. On this basis and from the isotope concentrations of the metabolic pools it is estimated that about one-sixth of the carbon atoms of the saturated liver fatty acids has been derived from pyruvic acid and about one-third from acetic acid; *i.e.*, the ratio of incorporation of pyruvic to acetic acid carbon atoms is about 1:2. This ratio which is independent of the half life time may probably be significant for the mechanism of fatty acid synthesis. In the present experiments acetic and pyruvic acids account for only about one-half of the carbon atoms of the saturated liver fatty acids. The remaining carbon atoms may have originated from such unlabeled sources as the carcass fatty acids or the dietary cod liver oil. On a completely fat-free diet a larger portion of the saturated liver fatty acids could possibly arise by synthesis.

The isotope concentrations of the unsaturated liver fatty acid have little significance in themselves because this fraction contains also the multiply unsaturated acids. The latter are essential; they are not synthesized in the animal (24) and therefore do not contain the label. The isotope concentration of the oleic acids will therefore be considerably higher than the total unsaturated fraction and may even approach the tracer concentration of the saturated fatty acids in the liver. However, the ratio of incorporation of pyruvic to acetic acid carbon into the unsaturated fatty acids can be determined. In the laboratory strain this ratio is about 4:5. It differs sufficiently from the ratio for the saturated acids to suggest a possible difference of the respective synthetic mechanisms (25).

A similar estimation of the incorporation of pyruvic acid carbon into the liver fatty acids of the Sprague-Dawley rats cannot be carried out in view of the conversion of pyruvic acid to acetic acid in these animals. However, the comparison of isotope ratios gives some indirect information. In Experiment 4 (Table IV) in which pyruvic and acetic acids had been fed to the same rat, the ratio for liver cholesterol is

$$\frac{CC \text{ (pyruvic acid)}}{CC \text{ (acetic acid)}} = 0.25$$

for the saturated liver fatty acids (Table V) it is

$$\frac{CC \text{ (pyruvic acid)}}{CC \text{ (acetic acid)}} = 0.5$$

and for the unsaturated liver fatty acids (Table V) it becomes

$$\frac{CC \text{ (pyruvic acid)}}{(CC \text{ acetic acid})} = 1$$

The higher ratios found in the liver fatty acids are most readily interpreted by assuming direct incorporation of pyruvic acid carbon atoms. If the labeled carbon of pyruvic acid were to enter the isolated compounds only after conversion of pyruvic acid into acetic acid, an identical ratio in all metabolites would have to be expected.

In both strains, the total and the saturated carcass fatty acids are found to have approximately identical isotope concentrations. As the carcass fat contains only a small quantity of multiply unsaturated fatty acids (26), the oleic acid fraction will have about the same isotope concentration as the saturated fatty acids. These results are in contrast to the experiments carried out with heavy water (27) in which the deuterium concentration of the unsaturated fraction is considerably lower than that of the saturated fatty acids. This difference is not due to the use of growing animals, because in the experiment with an adult rat under identical conditions the total and saturated fatty acids also have identical isotope contents. It seems most plausible to attribute the variations of experiments with labeled hydrogen and labeled carbon to different mechanisms of synthesis of saturated and unsaturated fatty acids (25). Such differences of the mechanisms were indicated by the unequal ratios of incorporation of acetic and pyruvic acid carbon atoms into saturated and unsaturated liver fatty acids and might easily account for the unequal uptake of deuterium from the body water.

DISCUSSION

Considerable differences were observed after feeding labeled pyruvic acid to two strains of rats, the laboratory strain and the Sprague-Dawley strain. In contrast the acetic acid metabolism proved to be nearly identical in both. The differences observed in the two strains can be most readily accounted for, if the assumption is made that the Sprague-Dawley rats convert pyruvic acid to acetic acid on a high carbohydrate diet, whereas the laboratory rats do not do so. In this connection the observation by Krahl and Cori (28) may be relevant. These authors found that Sprague-Dawley rats are much more resistant to the production of alloxan diabetes, as measured by the increase of the blood glucose level, than another strain. It may perhaps be speculated that this observation can be attributed to two competing pathways of pyruvic acid metabolism; namely, conversion to acetic acid and to glucose respectively.

Experiments with heavy water by Schoenheimer *et al.* had shown that on a high carbohydrate diet the depot fat turns over at a rate requiring the daily synthesis of considerable quantities of fatty acids from small molecules. From similar heavy water experiments Stetten and Boxer (2) concluded that 35 per cent of dietary glucose is metabolized by way of fatty acids. However, the experiments with isotopic carbon reported here lead to the conclusion that the conversion of carbohydrate to fat by way of pyruvic acid accounts for a small fraction only of the metabolism of dietary carbohydrate. The fate of the major part of carbohydrate metabolism remains unaccounted for.

In the laboratory strain, pyruvic acid accounts for only about one-third of the carbon atoms of the saturated and for about four-ninths of the unsaturated liver fatty acids synthesized. The other carbon atoms are provided by acetic acid. In the Sprague-Dawley strain the metabolic sequence

$$\begin{tikzcd}\text{Carbohydrate} \arrow[r] & \text{pyruvic acid} \arrow[r] \arrow[rr, bend right] & \text{acetic acid} \arrow[r] & \text{fatty acids}\end{tikzcd}$$

may account for a somewhat larger part of carbohydrate metabolism.

The author is indebted to Mrs. Ruth Raper for valuable assistance in the course of this work.

SUMMARY

Carbonyl- and carboxyl-labeled pyruvic acids and carboxyl-labeled acetic acid were synthesized and fed to two strains of rats.

Acetyl derivatives of *p*-aminobenzoic and γ-phenylaminobutyric acids were extracted from the urine; glycogen, cholesterol, and fatty acid were isolated from the liver and cholesterol and fatty acid from the carcass.

In the Sprague-Dawley strain conversion of pyruvic acid to acetic acid seems to occur.

In the laboratory strain no significant conversion of pyruvic acid to acetic acid was found. An estimate of the pyruvic acid pool could be made, indicating that pyruvic acid is not in complete equilibrium with dietary carbohydrate. Pyruvic and acetic acid carbon atoms are incorporated into fatty acids. On a quantitative basis acetic acid provides a larger number of carbon atoms for the fatty acids than pyruvic acid.

The utilization of pyruvic acid as a precursor for cholesterol is small.

BIBLIOGRAPHY

1. Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **114**, 381 (1936).
2. Stetten, D., Jr., and Boxer, G. E., *J. Biol. Chem.*, **155**, 231 (1944).
3. Stetten, D., Jr., and Boxer, G. E., *J. Biol. Chem.*, **156**, 271 (1944).
4. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **145**, 625 (1942).
5. Rittenberg, D., and Bloch, K., *J. Biol. Chem.*, **160**, 417 (1945).
6. Smedley, I., and Lubrzynska, E., *Biochem. J.*, **7**, 364 (1913).
7. Anker, H. S., *J. Biol. Chem.*, **176**, 1333 (1948).
8. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **15**, 311 (1913).
9. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **159**, 45 (1945).
10. Shemin, D., *J. Biol. Chem.*, **162**, 297 (1946).
11. Schoenheimer, R., and Dam, H., *Z. physiol. Chem.*, **215**, 59 (1933).
12. Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **113**, 505 (1936).
13. Piper, S. H., Pollard, A., Andrews, J., Smith, B., and Williams, F., *Biochem. J.*, **25**, 2072 (1931).
14. Reid, A. F., in Wilson, D. W., Nier, A. O. C., and Reimann, S. P., Preparation and measurement of isotopic tracers, Ann Arbor, 83 (1946).
15. Rittenberg, D., in Wilson, D. W., Nier, A. O. C., and Reimann, S. P., Preparation and measurement of isotopic tracers, Ann Arbor, 31 (1946).
16. Rittenberg, D., and Foster, G. L., *J. Biol. Chem.*, **133**, 737 (1940).
17. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **155**, 243 (1944).
18. Knoop, F., *Beitr. chem. Physiol. u. Path.*, **6**, 150 (1905).
19. Vennesland, B., Solomon, A. K., Buchanan, J. M., Cramer, R. O., and Hastings, A. B., *J. Biol. Chem.*, **142**, 371 (1942).
20. Mackenzie, C. G., and du Vigneaud, V., *J. Biol. Chem.*, **172**, 353 (1948).
21. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 671 (1941).

22. Chargaff, E., and Sprinson, D. B., *J. Biol. Chem.*, **148**, 249 (1943).
23. Buchanan, J. M., Sonne, J. C., and Delluva, A. M., *J. Biol. Chem.*, **173**, 81 (1948).
24. Bernhard, K., and Schoenheimer, R., *J. Biol. Chem.*, **133**, 707 (1940).
25. Bernhard, K., and Bullet, F., *Helv. chim. acta*, **26**, 1185 (1943).
26. Hilditch, T. P., The chemical constitution of natural fats, New York (1941).
27. Rittenberg, D., and Schoenheimer, R., *J. Biol. Chem.*, **121**, 235 (1937).
28. Krahl, M. E., and Cori, C. F., *J. Biol. Chem.*, **170**, 607 (1947).

ON THE METABOLIC FATE OF PYRUVAMIDE AND ACETAMIDE*

By H. S. ANKER AND R. RAPER

(*From the Department of Biochemistry, University of Chicago, Chicago*)

(Received for publication, July 12, 1948)

The method used for the synthesis of labeled pyruvic acid as reported in the preceding communication (1) involved the isolation of labeled pyruvamide as an intermediate and suggested an investigation of the fate of this compound in relation to pyruvic acid. Pyruvamide showed a marked difference from pyruvic acid in its metabolic behavior. In addition, an experiment with labeled acetamide was carried out for comparison.

EXPERIMENTAL

The synthesis of pyruvamide and the isolation of body constituents were carried out as previously described (2).

Acetamide—Carboxyl-labeled potassium acetate was converted to acetyl bromide (1) and this compound added to an excess of liquid ammonia. After evaporation of the excess ammonia, the residue was extracted with hot ethyl acetate from which acetamide crystallized out after cooling. Yield, about 50 per cent; m. p. 78–79°.

Formic Acid—The urine was acidified with sulfuric acid, extracted with ether for 12 hours, and the ether extract evaporated to dryness. The residue was taken up in water, acidified, and treated with dinitrophenylhydrazine. The hydrazones were extracted with ethyl acetate and discarded. The remaining water layer was distilled with steam. The distillate was neutralized with sodium hydroxide, concentrated to a volume of 5 ml., and filtered. The *p*-bromophenacyl ester was then prepared as described by Hurd and Christ (3).

Results

Pyruvamide Feeding—Data showing the incorporation of isotopic carbon into a number of metabolites after pyruvic acid, pyruvamide, and acetic acid feedings to rats of a laboratory strain are presented in Table I. The significance of the isotope concentrations obtained in the various isolated compounds after administration of pyruvic and acetic acids has been discussed previously (2). In particular it was found that in the laboratory strain only a negligible amount of pyruvic acid is converted to acetic acid.

---

* This work was supported by a grant from the United States Public Health Service.

It can be seen by inspection of Table I that in all isolated compounds the distribution of the labeled carbon from pyruvamide is practically identical to that found after the administration of acetic acid. The results, however, differ significantly from those obtained after feeding pyruvic acid. Pyruvamide in contrast to pyruvic acid is a source of acetyl groups for acetyl-*p*-aminobenzoic acid exactly as is acetic acid. Pyruvic acid contributes less labeled acetyl for the acetylation of γ-phenylaminobutyric acid than does either acetic acid or pyruvamide. The isotope contents of glycogen and cholesterol, isolated from the liver, are similar after pyruvamide and acetic acid feeding but differ distinctly from that obtained after administration of pyruvic acid. In experiments in which pyruvic acid and pyruvamide were injected rather than fed, a similar pattern of distribution of the label was obtained. These results may be taken as evidence that pyruvamide undergoes splitting between carbon atoms 1 and 2 in the body and that this splitting reaction occurs at a considerably faster rate than hydrolysis to pyruvic acid. The possibility was tested that the splitting of pyruvamide yields, besides acetic acid, a fragment consisting of the carboxyl carbon and amide nitrogen, which could contribute to the synthesis of urea. Carboxyl-labeled pyruvamide was therefore fed. The results shown in Table II indicate that the isotope concentration of urea did not differ from that of the expired carbon dioxide. It is therefore unlikely that the carboxyl carbon of pyruvamide is a specific precursor of urea. The analytical values from this experiment were considerably

TABLE I

Isotope Concentrations of Body Constituents after Feeding Labeled Pyruvic Acid, Pyruvamide, and Acetic Acid

Compounds isolated	Relative isotope concentrations* after feeding†		
	Pyruvic acid‡	Pyruvamide‡	Acetic acid
	per cent	*per cent*	*per cent*
Acetyl group of acetyl-*p*-aminobenzoic acid	0.10	2.1	3.5
" " " acetylphenylaminobutyric acid	1.3	2.6	2.7
Liver glycogen	0.12	0.05	0.03
" cholesterol	0.26	0.54	0.66
" saturated fatty acids	0.73	0.66	0.71

$$* \ RIC = \frac{\text{specific radioactivity of isolated compound}}{\text{specific radioactivity of fed compound}}.$$

† 0.46 mM per day for 3 days.

‡ Radioactivity of pyruvamide and pyruvic acid calculated as present in 2 carbon atoms only.

below the isotope concentration of urea isolated after the administration of carboxyl-labeled pyruvic acid. This indicates that carbon atom 1 of pyruvamide is not readily converted to CO_2. From the urine of Sprague-Dawley rats fed normal pyruvamide, formic acid could be isolated and identified in the form of its *p*-bromophenacyl ester. Sonne *et al.* (4) have shown that formic acid is not converted to carbon dioxide in the pigeon. The occurrence of formic acid in the urine after pyruvamide feeding together with the low isotope concentration of carbon dioxide after feeding carboxyl-labeled pyruvamide suggests that the splitting of pyruvamide in the body yields acetic and formic acids as products.

Pyruvamide in Liver Slices—The utilization of carbon atoms of pyruvamide for the synthesis of fatty acids and cholesterol *in vitro* was determined under conditions which permit the demonstration of fatty acid synthesis in liver slices.[1] The data are given in Table III.

TABLE II

Isotope Concentration of Urea and Respiratory Carbon Dioxide after Feeding Carboxyl-Labeled Pyruvamide

	Isotope concentrations after feeding carboxyl-labeled pyruvamide*
	atom per cent excess C^{13}
CO_2 after 12 hrs.	0.03
" " 30 "	0.03
" " 52 "	0.00
Urea, 1st 30 hrs.	0.03
" 2nd 22 "	0.00

* 51 per cent excess C^{13} in the carboxyl carbon.

Bloch has shown that in this system the synthesis of cholesterol from labeled acetic acid in the presence of insulin is not materially changed by the addition of pyruvic acid. It can be seen from Table III that in the presence of pyruvic acid the isotope is incorporated to a similar extent from labeled pyruvamide or labeled acetic acid into the isolated cholesterol. If both labeled acetic acid and unlabeled pyruvamide are present, the isotope concentration of the cholesterol is lower. If it is assumed that acetic acid and pyruvamide can be utilized interchangeably, as can be concluded from the fact that individually both are utilized to a similar extent for cholesterol synthesis, the lower isotope value will be due to dilution of the labeled acetic acid by carbon atoms derived from the unlabeled pyruvamide. The experimental data are consistent with this assumption.

---

[1] Bloch, K., private communication.

Bloch and Kramer (5) have also shown that in the presence of insulin the incorporation of isotope into the fatty acids from labeled acetic acid is considerably enhanced if unlabeled pyruvic acid is added to the medium. The data in Table III indicate that pyruvamide can, at least partially, replace acetic acid as a source of carbon atoms for the synthesis of fatty acids. If in the experiments with labeled acetic acid normal pyruvamide is substituted for pyruvic acid, only a negligible uptake of labeled carbon into the fatty acids is observed.

TABLE III

Isotope Concentrations of Fatty Acids and Cholesterol after Addition of Labeled Pyruvamide and Acetic Acid to Rat Liver Slices

1.5 gm. of liver slices in 16 ml. of Krebs-Ringer bicarbonate buffer at pH 7.4, containing 0.8 unit of insulin per ml. Incubated for 3 hours at 37° in O_2-CO_2.

Compounds isolated	Isotope concentrations* after addition of		
	0.12 mM 2-C^{14} pyruvamide† and 0.18 mM pyruvic acid	0.12 mM 1-C^{14} acetic acid and 0.18 mM pyruvic acid	0.12 mM 1-C^{14} acetic acid and 0.18 mM pyruvamide
	per cent	*per cent*	*per cent*
Fatty acids	0.07	0.18	0.008
Cholesterol	0.8 ± 0.3	0.7 ± 0.1	0.24

* Per cent specific radioactivity of added compounds.

† Radioactivity of pyruvamide calculated as present in 2 carbon atoms only.

TABLE IV

Isotope Concentrations after Administration of Labeled Acetic Acid and Acetamide

Compounds isolated	Relative isotope concentrations* after feeding†	
	Acetic acid	Acetamide
	per cent	*per cent*
Acetyl group of acetyl-*p*-aminobenzoic acid	4.4	2.0
Liver cholesterol	0.73	0.07
" saturated fatty acids	1.6	0.16
Urea	0.49	0.15

$$* \; RIC = \frac{\text{specific radioactivity of isolated compound}}{\text{specific radioactivity of fed compound}}$$

† 0.46 mM per day for 3 days.

These results are in accord with those obtained in the feeding experiments in showing that pyruvamide *in vitro* acts similarly to acetic acid but differs considerably from pyruvic acid. In this series of experiments too, pyruvamide seems to be split much faster than it is hydrolyzed.

Acetamide Feeding—The isotope concentrations obtained after feeding labeled acetamide and labeled acetic acid to Sprague-Dawley rats are given in Table IV. It can be seen that acetamide is only about one-tenth as effective as acetic acid as a precursor for fatty acids and cholesterol. This finding indicates that only a small fraction of acetamide carbon enters the acetic acid pool. The hydrolysis to acetic acid is probably a slow process.

On the other hand, however, acetamide is quite efficiently used for the acetylation of *p*-aminobenzoic acid, a value of about one-half of the isotope concentration of acetic acid being obtained. As the low value of isotope incorporation into fatty acids and cholesterol excludes the fact that acetic acid is formed in large amounts from acetamide, the assumption may be made that acetamide is capable of acetylating *p*-aminobenzoic acid directly, *i.e.* without prior hydrolysis.

SUMMARY

Evidence has been presented to indicate that in the intact animal pyruvamide is split into acetic acid and formic acid, rather than hydrolyzed to pyruvic acid. The splitting reaction must occur at a fast rate. In liver slices pyruvamide can substitute for acetic acid as precursor for cholesterol and fatty acids. The significance of pyruvamide as a normal metabolite is unknown.

Acetamide is only partially and slowly hydrolyzed to acetic acid. The possibility exists that it may acetylate *p*-aminobenzoic acid without being hydrolyzed.

BIBLIOGRAPHY

1. Anker, H. S., *J. Biol. Chem.*, **176**, 1333 (1948).
2. Anker, H. S., *J. Biol. Chem.*, **176**, 1337 (1948).
3. Hurd, C. D., and Christ, R. E., *J. Am. Chem. Soc.*, **57**, 2007 (1935).
4. Sonne, J. C., Buchanan, J. M., and Delluva, A. M., *J. Biol. Chem.*, **173**, 69 (1948).
5. Bloch, K., and Kramer, W., *J. Biol. Chem.*, **173**, 811 (1948).

NAPHTHOQUINONE ANTIMALARIALS

XXI. ANTISUCCINATE OXIDASE ACTIVITY*

BY HANS HEYMANN† AND LOUIS F. FIESER

(*From the Chemical Laboratory, Harvard University, Cambridge*)

(Received for publication, May 10, 1948)

Ball, Anfinsen, and Cooper[1] studied a few representative 2-hydroxy-3-alkyl-1,4-naphthoquinones under investigation as antimalarial drugs with results that indicated a probable parallelism between *in vivo* activity in the inhibition of respiration of succinate oxidase and antimalarial activity as measured by assays in ducks. With kind guidance from Dr. Ball, we installed his test procedure and have investigated the possible parallelism in further detail.

EXPERIMENTAL

The inhibitory effect of naphthoquinones on mixtures of cytochrome *c* and dehydrated succinate oxidase from beef heart in a phosphate buffer containing sodium succinate was studied manometrically according to Ball's technique.[1] The compounds studied will be indicated by their code numbers; the structures are given in Table I. A typical dehydrated enzyme preparation at a concentration of 900 mg. per liter respired at the rate of 107 c.mm. per 30 minutes when freshly prepared, and at the rate of 86 c.mm. per 30 minutes 1 month later; as the aging progressed, more and more drug was required to produce 50 per cent inhibition of the oxygen consumption (*e.g.*, 0.54 mg. of M-1916 per liter instead of the original 0.46 mg. per liter). This effect seems attributable to the antagonism to drug action exerted by the increased amounts of enzymatically inert protein in the aged preparations. Thus portions of an old, completely inactive enzyme preparation added to the test solutions of a fresh preparation and standard drug (M-1916) caused a progressive increase in the drug required to half inhibit the system, and added plasma protein exerts a similar antagonism. However, neither the variation in a given enzyme preparation with time nor the variation from preparation to preparation was found to interfere with the determination of the potencies of a series of naphthoquinones relative to that of the standard, M-1916. When the inhibitory effect of a given compound was studied, a parallel determination was always made of the effect of M-1916 on the same enzyme preparation, and a blank was run with each set of concentrations to offset variation in the concentra-

---

* This work was done in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University, and in part with the aid of a grant from the Rockefeller Foundation.

With the technical assistance of Louise Wiarda and Jean B. Knowlton.

Papers I to XVII, *J. Am. Chem. Soc.*, **70**, 3151–3244 (1948); Papers XVIII to XX, *J. Pharmacol. and Exp. Therap.*, **94**, 85–124 (1948).

† Present address, Department of Chemistry, University of Oregon, Eugene, Oregon.

[1] Ball, E. G., Anfinsen, C. B., and Cooper, O., *J. Biol. Chem.*, **168**, 257 (1947).

TABLE I

Relative Antirespiratory Activity in Inhibition of Succinate Oxidase (37°)

Standard, M-1916 = 1.

Code No.	2-Hydroxy-1,4-naphthoquinone, side chain	Relative potency, weight basis	Activity against *P. lophurae*, ED_{95}
M-2261	—$(CH_2)_3$-α-Acenaphthyl	9.7	
M-295	—$(CH_2)_3$-β-Tetralyl	6.6	39.3
M-2254	—$(CH_2)_3$-α-Naphthyl	5.3	>20
M-2255	—$(CH_2)_3$-9-(1,2,3,4-Tetrahydrophenanthryl)	5.1	>80
M-2243	—$CH_2CH(CH_3)CH_2$-Cyclohexyl	4.4	17.4
M-297	—$(CH_2)_3$-β-Decalyl	4.3	8.6
M-333	—$CH_2CH(CH_3)(CH_2)_4CH(CH_3)_2$	4.0	7.7
M-1971	—$(CH_2)_4$-Cyclohexyl	3.8	11.4
M-289	—$(CH_2)_8CH{=}CH_2$	3.8	14.3
M-2257	—$(CH_2)_3$-5-Hydrindyl	3.1	34.7
M-273	—$C_{10}H_{21}$-*n*	2.6	20.4
M-285	—$CH_2CH(CH_3)$—C_6H_{13}-*n*	2.5	4.4
M-287	—$(CH_2)_5CH(CH_3)_2$	2.2	16.4
M-374	—$(CH_2)_3$-Δ^2-Cyclohexenyl	1.7	23.6
M-1714	—$C_{15}H_{31}$-*n*	1.2	43
M-1936	—$(CH_2)_{12}$-Cyclopentyl	1.2	87
M-2246	—$(CH_2)_2CH(CH_3)$-Cyclohexyl	1.1	39
M-1916	—$(CH_2)_3$-Cyclohexyl	1.0	21.4
M-2237	—$(CH_2)_3$—$C_6H_4(CH_3)_2$-2,5	0.73	>75
M-1933	—$(CH_2)_2CH(CH_3)(CH_2)_3CH(CH_3)_2$	0.59	13.9
M-1929	—$(CH_2)_4CH(CH_3)_2$	0.56	16.1
M-1944	—$CH(CH_3)(CH_2)_3CH(CH_3)_2$	0.35	5.6
M-2262	—$CH(CH_3)(CH_2)_2$-Cyclohexyl	0.13	
M-1955	—$(CH_2)_3C_6H_5$	0.11	65
M-1711	—$(CH_2)_3CH(CH_3)_2$	0.07	75
M-2263	—CH_2-Menthyl (Isomer B)	0.07	
M-1963	" (" A)	0.05	>100
M-2264	—Phytyl	0.04	
M-1523	—$CH_2CH_2CH(CH_3)_2$	0.03	68
M-1935	—C_6H_4Br-*p*	0.02	50

tion or activity of the different enzyme suspensions. The naphthoquinones give typical dosage-response curves that are linear up to about 70 per cent inhibition and then level off asymptotically. The drug activity is expressed as the concentration (by weight) necessary to cause 50 per cent inhibition of respiration (LD_{50}). The relative potency is given by the ratio of the LD_{50} values found for M-1916 and the substance studied in parallel determinations. In a series of comparisons in which the LD_{50} for M-1916 varied from 0.46 to 1.07 mg. per liter, the following values were found for the relative potency of M-297: 4.1, 4.5, 4.5, and 5.3.

Results

The activities found for twenty-nine naphthoquinones are listed in Table I in the order of decreasing relative potency. The *in vivo* activities against *Plasmodium lophurae* in ducks, as determined by A. P. Richardson, are reported in terms of the effective dose (ED_{95}) (mg. per kilo) required to produce a 95 per cent reduction in parasitemia (see Paper II). Some correlations between inhibitory power and antimalarial activity (last column) can be discerned in a sharply defined series of compounds; for example, among the compounds with normal and isoalkyl side chains the activity increases with increasing carbon content from C_5 to C_8 (0.03, 0.07, 0.56, 2.2) and decreases from a C_{10} chain (2.6) to a C_{15} chain (1.2). The peak of antisuccinate oxidase activity seems to be about the same as that for antimalarial activity, but beyond the peak the loss in activity with increasing molecular weight is less abrupt. The four compounds of highest antirespiratory activity all have side chains of high carbon content (C_{13} to C_{17}).

However, when the results are considered as a whole, certain glaring discrepancies are observed between the *in vitro* and *in vivo* activities. M-295 is the second most active inhibitor and is 6.6 times as potent as M-1916, but it is only half as active as an antimalarial; M-1944 is only one-third as effective an inhibitor as M-1916 but is nearly 4 times as active as this substance in the duck assays. As a further test of the validity of experimentation with this enzyme system, examination was made of several compounds differing significantly in structure from the hydroxyalkylnaphthoquinones that show characteristic antiplasmodial activity. It was found that a hydroxyl or carboxyl group in the side chain results in a practically complete loss in antirespiratory activity, as well as in antiplasmodial activity, and that the introduction of a methyl group into the benzenoid nucleus of an active hydroxyalkylnaphthoquinone destroys the antisuccinate oxidase activity, whereas methyl substitution in the side chain has the opposite influence. In these subtle effects activity in the inhibition of succinate oxidase parallels antimalarial activity. However, M-1923, the chloride of hydrolapachol (M-1523) was found to have an antirespiratory potency 4.5 times as great as hydrolapachol, and yet assays in ducks showed the compound to be completely inactive. Another probable discrepancy is that α-naphthoquinone showed marked activity as a respiration inhibitor, namely 46 per cent the potency of M-1916. It seems necessary to conclude that antirespiratory activity against the succinate oxidase system cannot be relied upon to give even an approximate index of antimalarial potency.

SUMMARY

Activity in the inhibition of the succinate oxidase system does not appear to provide a reliable guide to antimalarial activity *in vivo*.

NAPHTHOQUINONE ANTIMALARIALS

XXII. RELATIVE ANTIRESPIRATORY ACTIVITIES (PLASMODIUM LOPHURAE)*

BY LOUIS F. FIESER AND HANS HEYMANN†

(*From the Chemical Laboratory, Harvard University, Cambridge*)

(Received for publication, May 20, 1948)

In Papers XIX and XX of this series we have reported application of the procedure of Wendel[1] for determination of the activities of 2-hydroxy-3-alkyl-1,4-naphthoquinones in the inhibition of respiration of parasitized red blood cells to the study of naphthoquinone-protein interactions and to the investigation of metabolic drug deactivation. The present paper reports the results of the determination of the biological activities of an unusually extensive series of related compounds made available by a war time research.

Results

The methodology was that described in Paper XIX. Table I records the antirespiratory activities of several series of compounds relative to that of compound M-1916. The relative potency is reported on a molar basis as calculated from the ratio, IC_{50}^{m} (M-1916)/IC_{50}^{m} (quinone studied), where IC_{50}^{m} is the molar concentration required to reduce respiration by 50 per cent. When more than one determination was made, the average deviation is recorded and the number of determinations is given in parentheses. In some instances the values reported are averages of fourteen to twenty-three comparisons that were conducted over a period of several months in the course of studies of protein antagonism and drug metabolism, and the average deviation is in the order of 15 to 25 per cent. For comparison with the molar *in vitro* activities in the inhibition of respiration of parasitized red blood cells, figures are given in the last column of Table I for the molar *in vivo* activities against *Plasmodium lophurae* in the duck relative to

---

* This work was done in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University, and in part with the aid of a grant from the Rockefeller Foundation.

Papers I to XVII, *J. Am. Chem. Soc.*, **70**, 3151–3244 (1948); Papers XVIII to XX, *J. Pharmacol. and Exp. Therap.*, **94**, 85–124 (1948).

With the technical assistance of Shirley R. Katz and Sally S. Shy.

† Present address, Department of Chemistry, University of Oregon, Eugene, Oregon.

[1] Wendel, W. B., *Federation Proc.*, **5**, 406 (1946).

TABLE I

Relative Activity in Inhibition of Respiration of Parasitized Red Blood Cells at 41°
(Plasmodium lophurae in Ducks)

Standard, M-1916 = 1.

Code No.	Side chain, type	n	Relative molar activity	
			Antirespiratory*	*In vivo*
M-1709	$—(CH_2)_nCH_3$	3	0.05 ± 0.01 (3)	0.1
M-1710		4	0.15 ± 0.02 (2)	0.15
M-268		5	0.5 ± 0.02 (2)	0.2
M-260		6	0.5 ± 0.09 (3)	0.4
M-271		7	1.3 ± 0.1 (3)	0.6
M-2275		8	2.1 ± 0.1 (3)	2.5
M-273		9	1.65 ± 0.2 (4)	1.1
M-1926		10	3.1 ± 0.1 (5)	0.6
M-1928		11	2.5 ± 0.1 (3)	0.9
M-1924		12	4.6 ± 0.2 (2)	0.5
M-2347		13	1.9 ± 0.1 (3)	Feeble
M-1714		14	0.51 ± 0.05 (2)	0.6
M-1706	$—(CH_2)_nCH(CH_3)_2$	1	0.1	0
M-1523		2	0.3	0.3
M-1711		3	0.4	0.25
M-1929		4	1.4	1.2
M-287		5	1.7	1.25
M-2284		6	1.5	2.5
M-300		7	4.6	2.3
M-2287		8	3.2	1.8
M-1920	$—(CH_2)_n$-Cyclopentyl	1	0.5	0.2
M-2321		2	0.7	0.6
M-2322		3	1.3	1.2
M-2331		4	1.1	1.3
M-2335		5	1.6	1.8
M-1914	$—(CH_2)_n$-Cyclohexyl	1	1.4	0.35
M-1915		2	1.1	0.9
M-1916		3	1.00	1.00
M-1971		4	1.6 ± 0.1 (2)	2.0
M-1956		5	2.3	0.8
M-384	$—(CH_2)_n$-4′-Cyclohexylcyclohexyl-*cis*	1	4.6 ± 0.2 (2)	2.75
M-2329		2	8.7 ± 0.2 (2)	0.6
M-2291		3	7.1 ± 0.3 (2)	1.9
M-380	$—(CH_2)_n$-4′-Cyclohexylcyclohexyl-*trans*	1	4.2	3.8
M-2330		2	4.6	3.7
M-2292		3	6.5	3.8

TABLE I—*Continued*

Code No.	Side chain, type	*n*	Relative molar activity	
			Antirespiratory*	*In vivo*
M-297	—$(CH_2)_3$-β-Decalyl-*trans*		1.6 ± 0.3 (4)	2.9
M-2279	—$(CH_2)_3$-β-Decalyl-*cis*		4.1 ± 0.6 (5)	7.2
M-266	—Cyclohexyl		2.5 ± 0.36 (14)	2.3
M-2328	—β-Decalyl-*cis*		12.6 ± 2.0 (4)	4.4
M-2374	—β-Decalyl-*trans*		4.0 ± 0.2 (3)	1.8
M-2293	—4′-Cyclohexylcyclohexyl-*trans*		4.8 ± 1.0 (9)	36.2
M-1955	—$(CH_2)_nC_6H_5$	3	0.3	0.3
M-2286		4	0.2	0.6
M-2276		5	0.4 ± 0.04 (2)	0.7
M-2387		6	0.8 ± 0.04 (2)	
M-2386		7	1.6 ± 0.1 (3)	
M-2382		8	1.7 ± 0.2 (3)	
M-2301		9	3.6 ± 0.4 (8)	1.95
M-1738	—$(CH_2)_nC_6H_5Cl$-*p*	1	0	0.2
M-2289		2	0.7 ± 0.11 (14)	1.2
M-2260		3	0.8 ± 0.1 (2)	1.2
M-2340		5	2.5	0.9
M-2344		9	7.7 ± 0.2 (3)	3.1
M-2358	—$(CH_2)_nC_6H_5Br$-*p*	2	0.4	0.9
M-2341		5	2.9	<0.6
M-2362		9	7.4	2.5
M-2346	—$(CH_2)_2C_6H_4CF_3$-*m*		0.1	0.2
M-2365	—$(CH_2)_2C_6H_{10}CF_3$-3′		0.91 ± 0.2 (3)	0.7
M-2380	—$(CH_2)_nC_6H_4OC_6H_5$-*p*	1	0.14 ± 0.07 (5)	
M-2338		2	2.8 ± 0.1 (6)	0.85
M-2309		3	4.6 ± 0.8 (23)	1.75
M-2361		4	0.9 ± 0.07 (3)	Feeble
M-2345		5	2.5 ± 0.2 (4)	"
M-2360		9	2.7	1.0
M-2331	—$(CH_2)_8C(OH)(CH_3)_2$		0.2 ± 0.01 (2)	Weak
M-2343	—$(CH_2)_8C(OH)(C_4H_9\text{-}n)_2$		2.1 ± 0.5 (3)	2.0†
M-2376	—$(CH_2)_6C(OH)(C_6H_{13}\text{-}n)_2$		0.1 ± 0.02 (2)	<1.6†
M-2350	—$(CH_2)_5C(OH)(C_5H_{11}\text{-}n)_2$		3.4 ± 0.8 (16)	7.8†
M-2367	—$(CH_2)_8C(OH)(C_6H_{13}\text{-}n)_2$		1.3 ± 0.1 (2)	5.5†
M-2363	—$(CH_2)_nC_6H_4OCH_3$-*p*	4	0.3 ± 0.04 (3)	0.5
M-2357		5	0.8	1.4(?)
M-2334		9	2.6 (2)	0.2

TABLE I—*Concluded*

Code No.	Side chain, type	*n*	Relative molar activity	
			Antirespiratory*	*In vivo*
M-2233	$-(CH_2)_4CH(CH_3)CO_2H$		0	0
	$-CH_2CH(CH_3)(CH_2)_4CO_2H$		0	
M-2336	$-(CH_2)_3-C_6H_{10}OH$-4′		0.1 ± 0.01 (4)	
	Isomeric metabolite		0.08	
M-2342	$-CO(CH_2)_2C_6H_5$		0.01	0
M-2349	$-CO(CH_2)_3-C_6H_{11}$		0	
M-2352	$-(CH_2)_{10}OC_6H_5$		6.2 ± 0.15 (2)	0.9
M-2370	$-(CH_2)_{10}OC_6H_4C_6H_5$-*p*		0	0
M-2353	$-(CH_2)_7CH{=}CH_2$		1.3	1.7

* The figures in parentheses refer to the number of determinations.

† By intramuscular administration.

M-1916; the calculations are based upon the best available ED_{95c} (corrected) (effective dose required to produce 95 per cent reduction in parasitemia) or ED_{95} values recorded in Paper II.

The results indicate that the *in vitro* test affords a reliable means for the preliminary screening of new compounds. Among the 82 naphthoquinones studied, there is no instance of high *in vivo* activity that is not reflected in high potency in the *in vitro* test. No exact parallelism exists between the two sets of results, and indeed instances can be cited of wide divergences; for example, the *cis*-β-decalyl derivative M-2328 is 3 times as active *in vitro* as *in vivo*, whereas the *trans*-4′-cyclohexylcyclohexyl derivative M-2293 has an activity *in vitro* of only 4.8 as compared with the exceptional value of 36.2 in the duck assays. Nevertheless, even the exceptional M-2293 would have been recognized as a compound of interest from the observation of an *in vitro* activity 4.8 times that of M-1916. New compounds are now submitted for assay only if they exhibit high activity as respiration inhibitors.

Another important service of the antirespiratory activities is the disclosure of significant biological potency that sometimes is not revealed by bird assays conducted by the usual method of oral administration. Thus the tertiary alcohols M-2343, M-2350, and M-2367 proved to be only feebly active against *Plasmodium lophurae* when given orally, but the high *in vitro* activities prompted reassay by the intramuscular route, and the compounds were found to possess high *in vivo* potency. The failure of these and other substances of high molecular weight and low solubility to produce any but a weak response in the oral assays very probably is attributable to faulty absorption; the *in vitro* test is thus of particular value because of the absence of the variable factor of absorption. A graphical

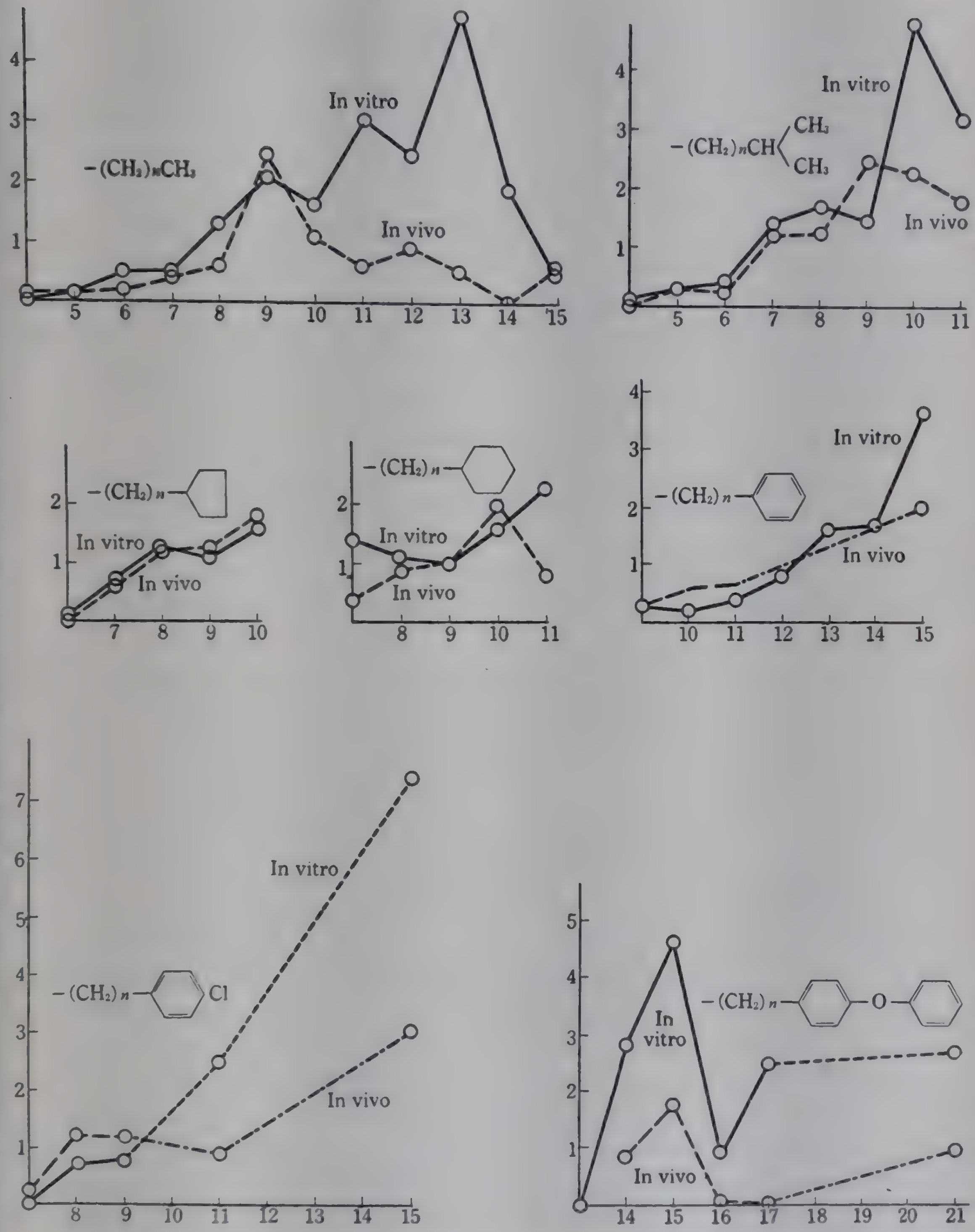


FIG. 1. Comparison of molar activities relative to M-1916 (ordinate), plotted against the number of carbon atoms in the side chain (abscissa).

comparison of the relative molar *in vivo* and *in vitro* activities is given in Fig. 1 for seven series of naphthoquinones. The *in vivo* charts have a decidedly different character than those of Paper II in which the activities are expressed on an absolute weight basis; in the chart of relative molar activities for the *n*-alkyl series, for example, the peak for the C_9 side chain appears very much more pronounced and there is a less abrupt loss in activity from C_{10} on. Both sets of data are subject to considerable experimental error and to the uncertainties of biological variation; some of the *in vivo* activities are based upon uncorrected ED_{95} values observed in a single assay, and some of the *in vitro* activities represent the results of a single series of Warburg determinations. Within the rather wide limit of tolerances indicated, it can be concluded from the charts that when the naphthoquinone side chain contains no more than about 10 carbon atoms the relative *in vivo* and *in vitro* activities show considerable correspondence. With an increase in the size of the side chain beyond this limit the *in vitro* activity continues to rise, probably as long as the naphthoquinone sodium salt possesses adequate solubility, whereas the *in vivo* activity either falls off or increases to a lesser extent as the result of increasingly poor absorption from the gut. That members of the arylalkyl series having C_{15} to C_{21} side chains are surprisingly high in *in vivo* activity may be associated with the greater hydrophilic character of this type of side chain, particularly when it contains a halogen substituent.

The enhancement of both types of activity by a *p*-halo substituent is noteworthy. The activities of *p*-chloro- and *p*-bromophenyl derivatives parallel one another closely over a wide range of activity (0.2 to 7). The following comparisons can be made of the ratio of activity of a *p*-chloro compound to that of the *p*-bromo derivative: *in vitro*, 1.7, 0.9, and 1.0, average 1.2; *in vivo*, 0.4, 1.3, 0.9, and 1.2, average 0.95. Evidently chloro and bromo derivatives possess the same potency on a molar basis, and hence the chloro compounds would be given preference in practical therapy. One comparison of *in vivo* activities indicates that a *p*-iodo derivative corresponds to the chloro and bromo compounds in molar activity; two comparisons are in agreement in showing that *p*-fluorophenyl derivatives have only 40 per cent the molar potency of the chloro, bromo, and iodo analogues.

Although the seven charts of Fig. 1 of relative *in vitro* activity may seem to conform to widely varying patterns, only one of them, that for the *n*-alkyl series, is extensive and complete enough to form a satisfactory basis for judgment. In this series the activity rises slowly and steadily to a peak at C_9 and then increases further to progressively higher peaks at C_{11} and C_{13}. The curve for the isoalkyl series starts off in a similar manner but extend only through one substantial peak (at C_{10}); those for the two cycloalkylalkyl series may represent merely the first parts of the curves of the *n*-alkyl type. The curve for the ω-phenylalkyl series, complete from C_9 to C_{15}, shows an initial slow rise and then a surge to a possible peak; again there is a suggestion of conformance to the pattern of the *n*-alkyl series. The *p*-phenoxyphenylalkyl series can perhaps be regarded as one having only two lower members before a C_{15} peak is reached.

On the assumption that the incomplete charts represent fragments conforming approximately to the general pattern discernible in the *n*-alkyl series, it is understandable why an alternation in the *in vitro* activities of odd and even carbon homologues is observed in some series but not in others. Alternation occurs in the *n*-alkyl series, but only in the region C_8 to C_{14} and not among the lower members. In the isoalkyl series a striking alternation is observed among the C_9, C_{10}, and C_{11} derivatives. In the next four series of Fig. 1 the charts are incomplete in the region where alternation could be reasonably expected. The data for the *p*-phenoxyphenylalkyl series cover this favorable region, and striking alternation exists from C_{14} to C_{17}. Since the C_{21} homologue has an odd carbon side chain, similar to the peak C_{15} and C_{17} members, it may represent a further peak of the incomplete chart. The extremely potent C_{15} *p*-chlorophenyl compound may bear a similar relationship to the C_{11} homologue of its series. Another limited instance of alternation not shown in Fig. 1 is among the three *cis*-4′-cyclohexylcyclohexylalkyl derivatives, whose *in vitro* activities are 4.6, 8.7, and 7.1; in the trans series no alternation was observed. Parallel alternations in *in vivo* activity are apparent with the C_8 to C_{10} *n*-alkyl compounds and with the C_{14} to C_{16} *p*-phenoxyphenylalkyl derivatives, whereas an alternation in the opposite sense is indicated for the three *cis*-4′-cyclohexylcyclohexyl compounds. The alternation in the *in vitro* activities is more profound, and the effect is of so striking a magnitude as to be indicative of a real phenomenon. Perhaps the odd or even character of the side chain determines firmness of binding to proteins.

TABLE II

Relative Susceptibility to Human Protein Antagonism

Code No.	Side chain	Relative anti-respiratory activity (duck)	$\frac{IC_{50}^{\text{human}}}{IC_{50}^{\text{duck}}}$
M-1926	$—C_{11}H_{21}$-*n*	3.1	1.0
M-1928	$—C_{12}H_{23}$-*n*	2.5	0.71
M-1924	$—C_{13}H_{25}$-*n*	4.6	0.82
M-2347	$—C_{14}H_{27}$-*n*	1.9	0.97
M-2338	$—(CH_2)_2C_6H_4OC_6H_5$-*p*	2.8	0.47
M-2309	$—(CH_2)_3C_6H_4OC_6H_5$-*p*	4.6	4.1
M-2361	$—(CH_2)_4C_6H_4OC_6H_5$-*p*	0.9	0.41
M-2345	$—(CH_2)_5C_6H_4OC_6H_5$-*p*	2.5	0.73
M-384	$—CH_2$-4′-Cyclohexylcyclohexyl-*cis*	4.6	1.4
M-2329	$—(CH_2)_2$-4′-Cyclohexylcyclohexyl-*cis*	8.7	3.8
M-2291	$—(CH_2)_3$-4′-Cyclohexylcyclohexyl-*cis*	7.1	1.3

The experiments reported in Table II were made to see whether alternation could be detected in the antagonistic effect of human plasma proteins. The three series of homologues selected for study all exhibit striking alternation in antirespiratory activity in suspensions in duck serum. In the *n*-alkyl series such differences as were observed in the relative susceptibility to added human serum are within the limit of experimental error. In the other two series a striking and consistent alternation was observed; odd and even homologues vary in susceptibility in some instances by a factor of 10. In each case the compounds that possess the peak potencies are the ones most strongly antagonized by human plasma proteins. The relationship is unfortunate from a practical point of view and demonstrates the importance of considering all available criteria for drug evaluation. That naphthoquinones particularly potent as respiration inhibitors are also highly susceptible to deactivation by specific proteins, presumably by virtue of a strong binding to the protein, suggests that the inhibitory action involves competitive protein binding; the drug action may therefore consist in combination with, and deactivation of, a respiratory enzyme.

SUMMARY

1. Comparison of relative *in vivo* activities of 82 naphthoquinones with the relative antirespiratory activities of the compounds in suspensions of parasitized erythrocytes shows that the two manifestations of biological activity are not fully parallel but that the convenient *in vitro* test provides a reliable guide to members of the series likely to possess significant *in vivo* activity.

2. In some of the series investigated, alternations for odd and even carbon homologues are observable both in the *in vitro* activities and in relative susceptibilities to human protein antagonism. Since the homologues of highest potency are those of greatest susceptibility, drug action apparently consists in combination with a respiratory enzyme and resulting deactivation.

THE NICOTINIC ACID, RIBOFLAVIN, D-AMINO ACID OXIDASE, AND ARGINASE LEVELS OF THE LIVERS OF RATS ON A PROTEIN-FREE DIET*

By SAM SEIFTER, DAVID M. HARKNESS, LEONARD RUBIN, AND EDWARD MUNTWYLER

(From the Department of Biochemistry, Long Island College of Medicine, Brooklyn, New York)

(Received for publication, July 20, 1948)

It is well established that in states of inanition and during periods of restricted protein intake animals suffer a loss of tissue protein, but the significance of this loss with relationship to enzyme activities and vitamin concentrations of tissue has not been clearly elaborated. In this respect, however, Potter and Klug (1) reported that the livers of rats maintained on a diet high in carbohydrate and low in protein or a diet high in fat and low in protein possessed diminished succinoxidase activity and decreased capacity for the oxidation of octanoate and citrate. Axelrod, Swingle, and Elvehjem (2), on the other hand, have reported an increase in the succinoxidase activity of livers from rats maintained on a restricted food intake for 3 weeks.

Recently, in a more extensive study, Miller (3) reported that the activities of a number of enzymes, including catalase, alkaline phosphatase, xanthine dehydrogenase, and cathepsin, are decreased in the livers of rats fasted for a period of 7 days. The observed loss in enzyme activity apparently paralleled or exceeded the loss of liver protein, and accordingly this author concluded that the decrease in activity represented a loss of enzyme protein *per se*. Similar results were reported by Miller (4) with respect to rats maintained on a protein-free diet.

A more quantitative relationship has been shown to exist between protein intake and liver arginase activity by Lightbody and Kleinman (5). These authors demonstrated that the liver arginase activity was directly related to the protein intake and the length of time the animals were maintained on a particular dietary régime. This relationship was interpreted as expressing an adaptation of the arginase content to a need for the enzyme as determined by the amount of protein which the organism was required to metabolize. No attempt was made by these workers to correlate the observed changes with the concentration of liver protein.

---

* Aided by a grant from Wyeth, Incorporated, Philadelphia. A preliminary report of this work was presented before the American Society of Biological Chemists, Atlantic City, New Jersey, March 15, 1948 (Seifter, S., Harkness, D. M., Feldman, B., Rubin, L., and Muntwyler, E., *Federation Proc.*, 7, 187 (1948)).

A relationship likewise has been shown to exist between protein intake and the liver concentrations of certain B vitamins. Thus, Sarett and Perlzweig (6), Unna and coworkers (7), and Reisen, Schweigert, and Elvehjem (8) have shown that the liver riboflavin is decreased in rats maintained on a low protein diet. On the other hand, Flinn and coworkers (9) observed an increase in the liver concentrations of nicotinic acid, pantothenic acid, pyridoxine, biotin, and vitamin B_c in chronically undernourished animals.

The present investigation was undertaken to determine (*a*) the nature of the relationship existing among the enzyme activities, vitamin concentrations, and the protein levels of tissues, and (*b*) the effect that variations in the diet have on this relationship. More specifically, the study is concerned with the changes which occur in the D-amino acid oxidase and arginase activities and the riboflavin and nicotinic acid concentrations of the livers of rats maintained on a protein-free diet. These particular liver constituents were chosen for study because they include (*a*) an enzyme which requires a vitamin coenzyme, (*b*) an enzyme which requires no known vitamin coenzyme, and (*c*) two vitamins which are known to be components of various enzyme systems.

EXPERIMENTAL

Twenty-four male Wistar strain rats, between 16 and 23 weeks of age and weighing between 280 and 313 gm., were placed on a normal diet, adequate in all respects, for a period of 7 days. At the end of this time the animals were divided into three groups of eight animals each. Four animals in Group A were placed on a protein-free diet for a period of 7 days. In a similar manner four animals in Group B were maintained on the same protein-free diet for 14 days. In Group C, four animals were kept on the protein-free diet for 21 days. In each group the remaining four animals served as pair-fed controls.

The control diet consisted of commercial casein, 20 per cent; sucrose, 65 per cent; corn oil, 10 per cent; Salts 4 (10), 4 per cent; and dried brewers' yeast, 1 per cent. The protein-free diet was similar in composition except that the casein was replaced with an equal amount of sucrose. In addition all animals received daily 2 drops of a vitamin supplement which furnished the following water-soluble vitamins in the amounts indicated: thiamine 25 γ, riboflavin 30 γ, nicotinamide 25 γ, pyridoxine hydrochloride 25 γ, calcium pantothenate 200 γ, choline chloride 10 mg., and inositol 3 mg. Twice weekly all animals received 2 drops of a cod liver oil-vitamin E supplement which furnished 200 U. S. P. units of vitamin A, 20 U. S. P. units of vitamin D, and 1 mg. of α-tocopherol.

At the end of the indicated feeding period the animal was sacrificed by a sharp blow on the head; its liver was quickly excised and blotted free of adhering blood, and sampled immediately for the determinations outlined below. Water was determined by drying to constant weight at 103°, and fat by weight difference after extraction with ether. Nitrogen was determined on the dry, fat-free residue by the micro-Kjeldahl method. Glycogen content, determined on the livers of some rats as mentioned below, was measured by the method of Deane and his coworkers (11). Nicotinic acid was determined microbiologically with *Lactobacillus arabinosus* as described by Snell and Wright (12). Riboflavin was determined microbiologically with *Lactobacillus casei* as described by Snell and Strong (13). The microbiological procedures were carried out on an extract prepared by autoclaving a weighed portion of liver at 15 pounds for 30 minutes.

Arginase activity was measured on an aliquot of a liver homogenate equivalent to approximately 0.3 mg. of tissue. The method of Van Slyke and Archibald (14) was employed for the determination, the formed urea being determined colorimetrically by the method of Archibald (15). Activity is expressed in Van Slyke-Archibald units.

In order to obtain maximum arginase activity, the liver aliquot was homogenized in 0.05 M $MnSO_4$ prepared in 0.9 per cent NaCl. It was found that the arginase activity as determined was dependent upon the length of time that elapsed between the killing of the animal and the initiation of the activation with manganese and also on the duration of the activation period. In order to keep these factors constant, and thus to obtain results on a comparable basis, a strict time schedule was adopted wherein homogenization with the manganous sulfate solution was started 5 minutes after the death of the animal, and activation was allowed to continue for 10 minutes before incubation with the substrate.

D-Amino acid oxidase was determined by a modification of the method described by Rodney and Garner (16). The modified procedure involves the measurement of the amount of keto acid formed by a liver homogenate after incubation with DL-alanine at a concentration of 0.005 M. This method was checked against determinations made by measuring the oxygen uptake under similar conditions, and excellent correlation was found within a fairly narrow range of tissue concentration. The essential steps of the procedure are detailed below.

The test system, contained in a 125 ml. Erlenmeyer flask, consisted of the following materials in the amounts indicated: 0.5 ml. of 0.04 M sodium arsenite prepared in 0.11 M NaCl, 0.5 ml. of 0.04 M DL-alanine, 2.0 ml. of Krebs-Ringer solution from which the calcium had been omitted, and 1.0 ml. of a finely divided, well mixed, liver homogenate containing between 100 and 200 mg. of tissue. A blank determination was prepared in the same manner except that the DL-alanine was replaced by an equivalent quantity of the Ringer's solution. The remainder of the determination was then carried out by the method of Sealock (17). The air phase above the mixture in the flask was replaced by oxygen, the flask was stoppered, and the system then allowed to incubate at 37° for exactly 6 hours. At the end of this time the reaction was stopped by the addition of 1.0 ml. of 20 per cent trichloroacetic acid, and 5 ml. of water were then added. The solution was filtered and the amount of keto acid formed was determined on 1 ml. of the filtrate by means of a 2,4-dinitrophenylhydrazone procedure. The D-amino acid oxidase activity is expressed in micromoles of pyruvate formed per unit of liver under these conditions.

TABLE I

Water, Fat, Glycogen, and Nitrogen Contents of Livers of Protein-Deficient and Control Rats

Group		No. of animals	Water	Fat	Glycogen	Nitrogen		
						On basis of fat-free solids	On basis of wet tissue	On basis of solids free of fat and glycogen
			gm. per kg.	*gm. per kg.*	*gm. per 100 gm.*	*gm. per kg.*	*mg. per gm*	*gm. per 100 gm.*
A	Protein-free, 7 days	4	708.6	24.2		9.45	25.20	
			(695–718)	(18–33)		(9.0–10.5)	(23.7–27.8)	
	Pair-fed control	4	697.4	15.7		11.29	32.25	
			(689–713)	(11–23)		(9.9–13.1)	(29.5–34.6)	
B	Protein-free, 14 days	4	687.1	58.1		8.60	21.85	
			(671–700)	(31–67)		(7.2–10.0)	(18.2–23.7)	
	Pair-fed control	4	698.9	11.5		11.82	34.14	
			(695–702)	(9–15)		(11.1–12.7)	(32.5–36.0)	
C	Protein-free, 21 days	8	668.4	80.0	9.1*	9.15	21.95	13.90*
			(636–694)	(45–122)	(8–10)	(7.9–10.5)	(20.8–24.6)	(12.9–14.9)
	Pair-fed control	8	696.3	16.4	3.8*	11.80	33.59	13.70*
			(692–703)	(9–29)	(2–5)	(10.4–12.7)	(30.1–36.8)	(13.3–14.0)

The figures in parentheses represent the range of the values.

* Four animals.

Results

Throughout the course of the study the experimental animals consumed an average of 11 gm. of food per day, and, on the average, lost 7 gm. in body weight per week. The animals on the control diet, which were pair-fed to the protein-restricted group, consumed an equal amount of food and gained, on the average, 3 gm. in body weight per week.

The results obtained from the analyses of the livers of the protein-restricted and control rats for water, fat, and nitrogen are presented in Table I. Maintenance on a non-protein diet, under conditions of adequate caloric intake, resulted in a decrease in the concentration of liver protein as indicated by the amount of nitrogen obtained per gm. of liver. The rate of decrease was greatest in the 1st week of protein restriction; however, the maximum absolute decrease was already apparent in the animals on the diet for 14 days. The experimental animals maintained for 21 days showed no further loss of liver protein concentration as compared with the 2 week group.

TABLE II

Nicotinic Acid and Riboflavin Concentrations and Arginase and D-Amino Acid Oxidase Activities of Livers of Protein-Deficient and Pair-Fed Control Rats

Group (4 animals each)		Nicotinic acid		Riboflavin		Arginase*		D-Amino acid oxidase activity†	
		γ per gm.	*mg. per gm. N*	*γ per gm.*	*mg. per gm. N*	*units per gm.*	*units × 10^{-3} per gm. N*	*units per gm.*	*units per gm. N*
A	Protein-free, 7 days	102.0 (95–104)	4.06 (3.8–4.4)	18.0‡ (17–19)	0.701‡ (0.66–0.77)	86§ (76–95)	3.6§ (3.1–4.0)	15.0 (12–20)	596 (489–805)
	Pair-fed control	152.8 (133–172)	4.75 (4.3–5.2)	26.6‡ (22–38)	0.824‡ (0.65–1.13)	434§ (426–503)	15.2§ (14.4–16.1)	34.6 (32–38)	1098 (923–1212)
B	Protein-free, 14 days	79.5 (62–99)	3.64 (3.0–4.2)	16.4‡ (13–18)	0.734‡ (0.71–0.78)	138 (35–263)	6.3 (1.9–12.8)	11.8 (8–15)	529 (395–646)
	Pair-fed control	139.1 (106–172)	4.08 (3.3–5.1)	35.3 (34–37)	1.037 (0.98–1.15)	560‡ (429–737)	16.4‡ (13.2–21.8)	39.2 (35–41)	1154 (970–1258)
C	Protein-free, 21 days	74.3 (65–81)	3.42 (3.1–3.9)	17.8 (13–22)	0.825 (0.60–1.04)	103‡ (54–243)	4.8‡ (2.5–11.6)	11.9 (10–15)	529 (437–653)
	Pair-fed control	147.2 (125–181)	4.58 (3.6–6.0)	33.1 (25–44)	1.015 (0.84–1.24)	383‡ (320–420)	12.1‡ (10.6–13.9)	43.1 (34–64)	1310 (980–1802)

* The unit is defined as the amount of arginase which in 1 minute at 25°, at pH 9.5, and substrate concentration of 0.285 M arginine will decompose 1 micromole of arginine to form 1 micromole of urea (see (14)).

† The activity is expressed here as units which are equivalent to the micromoles of pyruvate formed under the conditions of the assay.

‡ Three animals only.

§ Two animals only.

The diminution of liver protein was accompanied by a steady fall in the water content of the liver and a steady rise in the liver fat. As can be seen, the liver fat increased from about 1.5 per cent in the control animals to 8 per cent in the animals subsisting on the protein-free diet for 21 days.

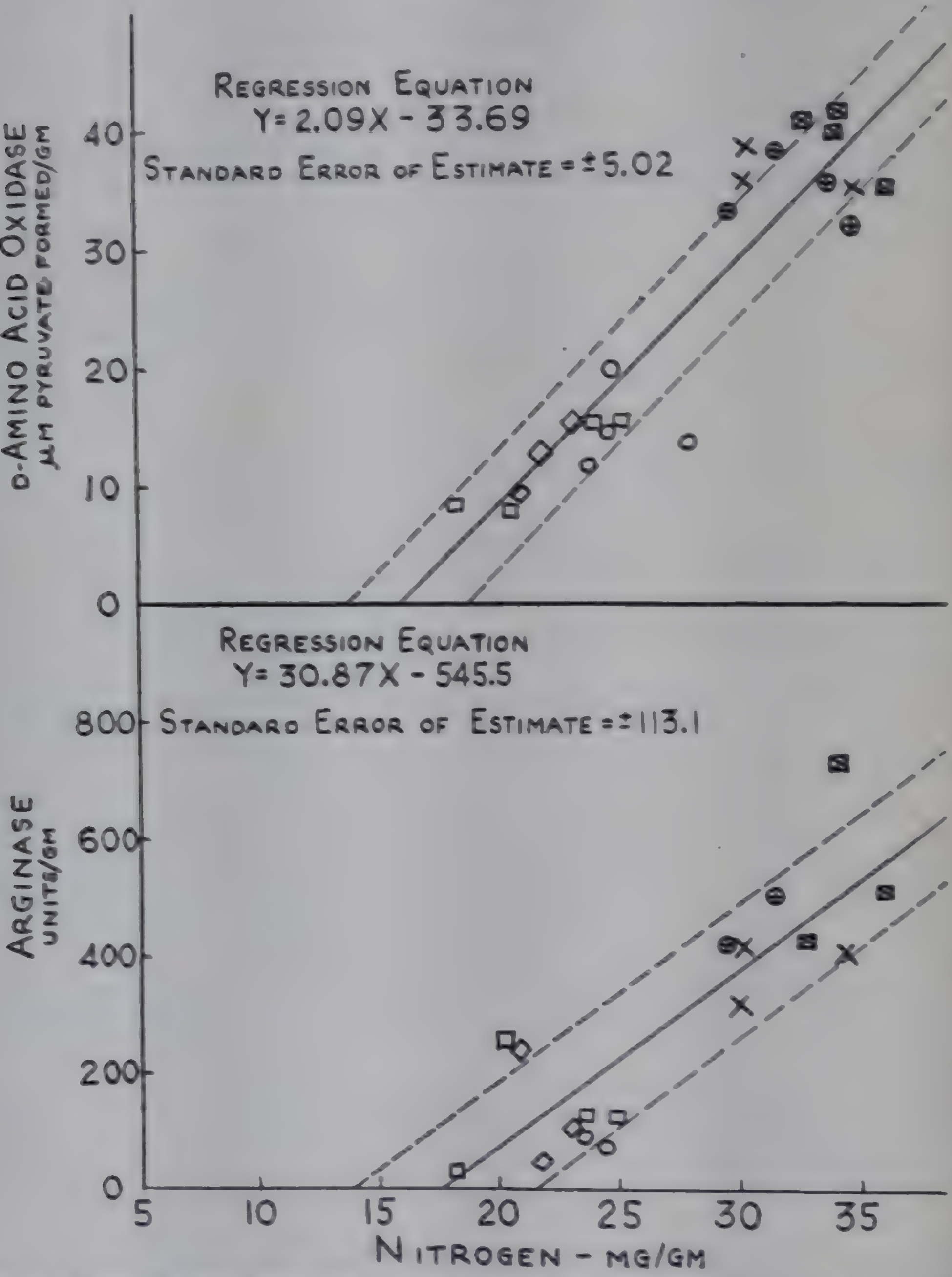


FIG. 1. Liver D-amino oxidase and arginase activities with respect to variations in liver nitrogen content. ○, Group A, protein-free diet; ⊖, Group A, control diet; □, Group B, protein-free diet; ⊠, Group B, control diet; ◇, Group C, protein-free diet; ×, Group C, control diet.

This change occurred even though all animals received 10 mg. of choline chloride per day.

The reduction in the nitrogen content of the fat-free liver solids in the groups of animals on the protein-free diet, which becomes apparent when comparison is made with the pair-fed controls, was most probably due to an increase in the liver glycogen. In evidence of this, Table I includes the values for liver glycogen of eight additional rats which were maintained on the same dietary régimes as the animals in Group C. The data show

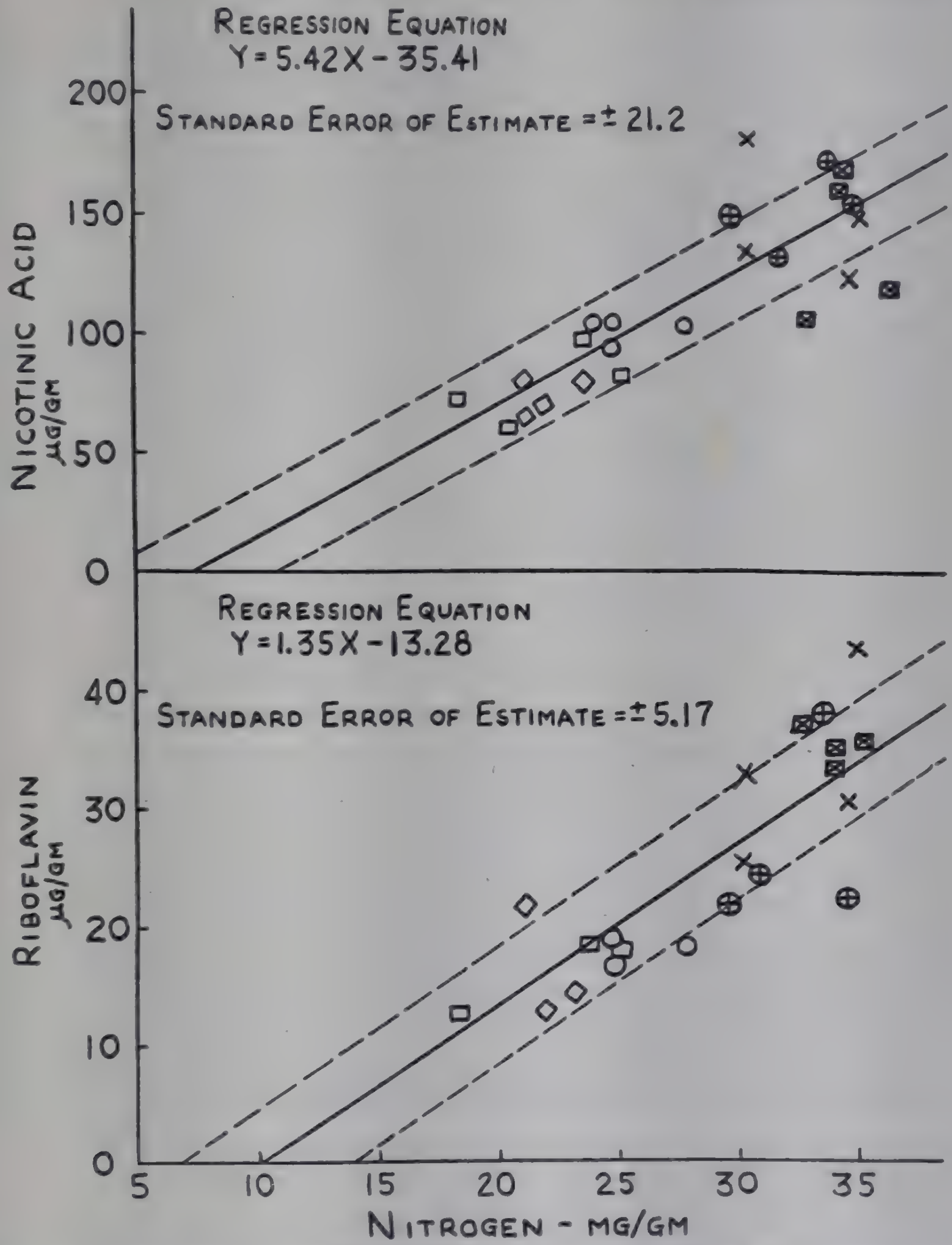


Fig. 2. Liver nicotinic acid and riboflavin concentrations with respect to variations in liver nitrogen content. For interpretation of reference points see the legend to Fig. 1.

that the animals on the protein-free, high carbohydrate diet had an average liver glycogen content of 9 per cent as compared with the figure of 4 per cent for the control group.

The results obtained from the analyses for riboflavin, nicotinic acid, D-amino acid oxidase, and arginase are presented in Table II. As in the case of the changes in protein concentration of the liver, protein restriction resulted in a decrease in the liver concentration of nicotinic acid and riboflavin as well as in the activities of the two enzymes. The diminution in the amounts or activities of these substances in all cases exceeded the decrease in the concentration of liver protein. This may be seen by comparing the values obtained per gm. of nitrogen in the protein-restricted groups with those obtained in the pair-fed controls. On this basis, the magnitude of decrease was considerably greater in the case of the enzymes than in the case of the vitamins.

The actual levels of the activities of D-amino acid oxidase and arginase were found to be directly related to the concentration of protein in the liver, as is shown in Fig. 1; here is shown the best straight line for the plot of enzyme activities against nitrogen per gm. of liver. From Fig. 1 it can be seen that a decrease in the liver protein concentration was accompanied by a decrease in the activities of these two enzymes. However, the rate of diminution of enzyme activity appears to have exceeded the rate of reduction in the liver protein as evidenced by the failure of the curve to go through the origin upon extrapolation to an enzyme activity of zero.

As is shown in Fig. 2, a similar type of relationship was observed to exist between the concentration of liver protein and the concentrations of riboflavin and nicotinic acid. As in the case of the enzymes, the levels of these two vitamins were directly related to the level of protein in the liver. This relationship was seen to hold, although the intake of these vitamins for all animals was maintained constant at a supposedly adequate level. Also, as was found with respect to the enzymes, the rates of decrease in the concentrations of riboflavin and nicotinic acid in the liver were greater than the rate of reduction in the protein concentration.

DISCUSSION

The increase in liver fat which, in the present study, was found to accompany protein restriction in the diet, is similar to the findings of Reisen and coworkers (8) for rats receiving a low protein diet and comparable amounts of choline. That such an increase is not due to a decreased intake of methionine *per se* was shown by these workers. In their experiments, for instance, the incorporation of this amino acid into the low protein diet at a level equivalent to the methionine content of a 20 per cent casein diet did not cause a lowering of the level of liver fat.

The decrease in the liver concentration of protein encountered in animals on a protein-free diet under conditions of adequate caloric intake is in marked contrast to the protein changes which apparently occurred in the livers of *fasted* rats as observed by Miller (3). For a given enzyme one may take the figure reported by this author for units of enzyme activity per gm. of liver and divide it by the reported figure for units of activity per gm. of liver protein. This result, divided by the factor 6.25, then yields a figure representing the concentration of nitrogen in the particular livers under consideration. Such an analysis of Miller's data shows an increase in the nitrogen concentrations of the livers of the fasted animals (36 mg. of nitrogen per gm. of liver) as compared with the control animals (32 mg. per gm. of liver).

The diminution in the activities of D-amino acid oxidase and arginase with dietary protein restriction is in agreement with the results obtained by Potter and Klug (1) with respect to the capacity of livers obtained from rats which had subsisted on a low protein diet to oxidize succinate, citrate, and octanoate, and, further, is consistent with the results of Miller (4), who found a similar decrease in the activities of five liver enzymes in animals fed a non-protein diet. The changes which have been reported with regard to the enzyme activities of the livers of fasted and undernourished animals (2, 3) would appear to indicate that the mechanisms involved in producing these changes differ from those operating in causing the changes observed in animals restricted only with respect to dietary protein. Furthermore, the direct relationship between enzyme activity and liver protein concentration, as found in the present study, does not necessarily hold in the case of fasting animals. Thus Miller (3) has found that the xanthine dehydrogenase and catalase activities of the livers of fasting animals may be found to decrease, even though appropriate calculations, as explained previously in this discussion, indicate that the protein concentration increases.

Since riboflavin is an integral part of the coenzyme necessary for the activity of D-amino acid oxidase and since the riboflavin concentration in the liver decreased on a non-protein diet, it would appear quite possible that the changes in D-amino acid oxidase activity observed in this study could be due to a decrease in coenzyme concentration. However, since a similar decrease was encountered in the activity of the enzyme arginase which does not require a vitamin cofactor for its activity, it is more likely that the diminution in activity of both enzymes represented a loss in enzyme protein *per se*. In this regard it is interesting to note that Lan

(18) has reported that rats bearing transplanted hepatomas showed a greater decrease in the activity of liver D-amino acid oxidase than of the liver concentration of the coenzyme.

The observed changes in the riboflavin and nicotinic acid concentrations in the liver would appear to indicate that the levels of these vitamins in the liver are independent of the intake over and above a certain minimum requirement, but rather depend largely on the level of tissue protein. In the case of riboflavin this observation is supported by similar findings which have been reported by other investigators (6–8).

The direct relationship which was found to exist between the concentration of liver protein and the levels of riboflavin and nicotinic acid suggests that these vitamins exist in a combined form in the liver and are not present as the free vitamins. Such an observation with respect to nicotinic acid has previously been made by Robinson and his coworkers (19).

SUMMARY

The effects of feeding rats a diet free of protein for varying periods of time have been studied with respect to the concentration of protein, riboflavin, and nicotinic acid, and the activities of D-amino acid oxidase and arginase in the livers of these animals.

Maintenance of rats on a protein-free diet caused a decrease in the concentration of liver nitrogen, a decrease in the liver water content, and an increase in the liver fat.

Animals subsisting on a protein-free diet possessed diminished liver arginase and D-amino acid oxidase activities and decreased liver concentrations of riboflavin and nicotinic acid as compared with pair-fed controls. These decreases exceeded the loss in liver nitrogen.

The activities of D-amino acid oxidase and arginase and the levels of riboflavin and nicotinic acid in the liver have been shown to be directly related to the concentration of liver nitrogen under the conditions of these studies.

The significance of these observations is discussed.

BIBLIOGRAPHY

1. Potter, V. R., and Klug, H. L., *Arch. Biochem.*, **12**, 241 (1947).
2. Axelrod, A. E., Swingle, K. F., and Elvehjem, C. A., *J. Biol. Chem.*, **145**, 297 (1942).
3. Miller, L. L., *J. Biol. Chem.*, **172**, 113 (1948).
4. Miller, L. L., *Federation Proc.*, **7**, 174 (1948).
5. Lightbody, H. D., and Kleinman, A., *J. Biol. Chem.*, **129**, 71 (1939).
6. Sarett, H. P., and Perlzweig, W. A., *J. Nutr.*, **25**, 173 (1943).

7. Unna, K., Singer, H. O., Kensler, C. J., Taylor, H. C., Jr., and Rhoads, C. P., *Proc. Soc. Exp. Biol. and Med.*, **55**, 254 (1944).
8. Reisen, W. H., Schweigert, B. S., and Elvehjem, C. A., *Arch. Biochem.*, **10**, 387 (1946).
9. Flinn, B. C., Pilgrim, F. J., Gregg, H. S., and Axelrod, A. E., *Proc. Soc. Exp. Biol. and Med.*, **63**, 523 (1946).
10. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **138**, 459 (1941).
11. Deane, H. W., Nesbett, F. B., and Hastings, A. B., *Proc. Soc. Exp. Biol. and Med.*, **63**, 401 (1946).
12. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
13. Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, **11**, 346 (1939).
14. Van Slyke, D. D., and Archibald, R. M., *J. Biol. Chem.*, **165**, 293 (1946).
15. Archibald, R. M., *J. Biol. Chem.*, **157**, 507 (1945).
16. Rodney, G., and Garner, R. L., *J. Biol. Chem.*, **125**, 209 (1938).
17. Sealock, R. R., *Science*, **94**, 73 (1941).
18. Lan, T. H., *Cancer Res.*, **4**, 37 (1944).
19. Robinson, J., Levitas, N., Rosen, F., and Perlzweig, W. A., *J. Biol. Chem.*, **170**, 653 (1947).

THE DEGRADATION OF L-LYSINE IN GUINEA PIG LIVER HOMOGENATE: FORMATION OF α-AMINOADIPIC ACID*

BY HENRY BORSOOK, CLARA L. DEASY, A. J. HAAGEN-SMIT, GEOFFREY KEIGHLEY, AND PETER H. LOWY

(From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena)

(Received for publication, June 21, 1948)

A summary of the little that is known of the metabolism of lysine in animals is as follows: it is indispensable in the diet, its α-amino group does not participate in reversible transamination reaction *in vivo* (2), neither the L nor D form is attacked by the appropriate amino acid oxidase, certain ε-nitrogen-substituted derivatives can replace lysine in the diet and their α-amino groups are oxidized by amino acid oxidases (3, 4), no α-nitrogen-substituted derivatives yet prepared can substitute for lysine in the diet (4–6).

Partly because so little was known, we have undertaken a study of the metabolism of lysine with the use of C^{14} as a tracer. The amino acid was synthesized with the isotope in the ε position and resolved into its L and D isomers. In order to observe in an initial exploration as many aspects of its metabolism as possible, one or the other isomer was made into a mixture with unlabeled amino acids, corresponding to the composition of casein, and incubated with guinea pig liver homogenate under different conditions.

The present communication deals with the finding of α-aminoadipic acid as a product of the degradation of lysine.

Preparations

Synthesis of C^{14}-Labeled Lysine—The steps in the synthesis are summarized in the following diagram; the position of the C^{14} is indicated by an asterisk.

$BaC^*O_3 \rightarrow C^*O_2 \rightarrow KC^*N \rightarrow NC^*\cdot CH_2\cdot CH_2\cdot CH_2Cl$ (γ-chlorobutyronitrile)
$\rightarrow NC^*\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH\cdot (COOC_2H_5)_2$ (Diethyl (3-cyano-*n*-propyl) malonate)
$\rightarrow NC^*\cdot CH_2\cdot CH_2\cdot CH_2\cdot C(=NOH)\cdot COOC_2H_5$ (ethyl 2-oximido-5-cyanovalerate)
$\rightarrow H_2N\cdot H_2C^*\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH(-NH_2)\cdot COOH$ (DL-lysine)

---

* This work is a part of that done under contract with the Office of Naval Research, United States Navy Department. A summary of this work has been reported (1).

The C^{14} used in this investigation was supplied by the Monsanto Chemical Company, Clinton Laboratories, Oak Ridge, Tennessee, and obtained on allocation from the United States Atomic Energy Commission.

KC^*N—309.8 mg. of BaC^*O_3 was converted to KC^*N by the method of Cramer and Kistiakowsky (7) and Loftfield (8, 9). The strongly alkaline solution obtained after decomposition of the excess potassium was concentrated to 4.7 gm. 90 per cent of the radioactivity was incorporated into the KC^*N.

*γ-Chlorobutyronitrile**—KCN was converted to γ-chlorobutyronitrile by a modification of the procedure previously described (10). To the KC^*N were added 1.253 gm. of inactive KCN dissolved in 4 ml. of H_2O and 28 ml. of absolute ethanol. The solution was titrated slowly at 0° to pH 10.8 with 6 N HCl; 3.8 ml. were consumed. The glass electrode was washed with 2 ml. of H_2O and the washings added to the solution, which was then refluxed with 19.2 gm. of trimethylene chlorobromide with vigorous stirring for $5\frac{1}{2}$ hours. After cooling, the solution was diluted with 45 ml. of water, and the two layers separated. To the lower layer was added a chloroform extract (12 ml.) of the top layer. The mixture was washed with 10 ml. of a 16 per cent $CaCl_2$ solution, then with 3 ml. of water, and dried over fused $CaCl_2$. The chloroform was removed by distillation at atmospheric pressure, and the residue distilled *in vacuo;* the product obtained at 89–93° and 24 mm. was used for the next step. Yield, 1.535 gm., or 75 per cent.

*Diethyl (3-Cyano-n-propyl) Malonate**—From the nitrile, DL-lysine was prepared by a modification of the method of Fischer and Weigert (11). 1.0 gm. of sodium in a Carius tube was dissolved in 10 ml. of absolute ethanol. After cooling there were added 13.3 gm. of diethyl malonate, then a mixture of 13.3 gm. of diethyl malonate and 2.74 gm. of chlorobutyronitrile*, followed by 13.3 gm. of diethyl malonate. The tube was sealed and heated for 15 hours at 95–98°. Ethanol, malonate, and remaining nitrile were removed by steam distillation, the residue extracted with ether, and the ether extract dried over potassium carbonate for 15 minutes, filtered, and distilled at 0.5 mm. Yield, 5.20 gm. (86 per cent); b.p. 128–131° at 0.5 mm.

*Ethyl 2-Oximido-5-cyanovalerate**—0.166 gm. of sodium was dissolved in 2.64 ml. of absolute ethanol and cooled to −18°. A mixture of 1.13 ml. of ethyl nitrite with 1.628 gm. of cold diethyl (3-cyano-*n*-propyl) malonate*, cooled to 0°, was then added dropwise into the ethylate solution and washed down with 0.66 ml. of cold absolute ethanol. After standing for 21 hours at −15° to −10°, the alcohol was removed at room temperature *in vacuo*. The residue, dissolved in 10 ml. of water, was extracted with 5 ml. of ether, and the ether washed with two 1 ml. portions of water. The combined aqueous solutions were cooled in ice, acidified with 10 per cent sulfuric acid, and extracted with ether. After removal of the solvent *in vacuo* and drying over sulfuric acid, the ester crystallized. Yield, 1.195 gm. (90.5 per cent).

DL-*Lysine**—In a flask with a reflux condenser 1.252 gm. of ethyl 2-oximido-5-cyanovalerate* were dissolved in 100 ml. of absolute ethanol, and 10 gm. of sodium added in small pieces as rapidly as possible. After 75 minutes the metal was almost completely dissolved. 10 ml. of water were added, and the solution refluxed for 45 minutes. It was then cooled in ice and acidified by adding 50 per cent H_2SO_4 slowly with vigorous stirring. The sodium sulfate was filtered off and washed several times by suspension in 95 per cent ethanol. Filtrate and washings were concentrated *in vacuo,* and the residual alcohol removed by steam distillation. The remaining aqueous solution was made alkaline to phenolphthalein with barium hydroxide, boiled, and the barium sulfate filtered off. The excess barium was removed with carbon dioxide and the filtrate concentrated to a syrup *in vacuo.* The syrup was taken up in ethanol, and a 5 per cent alcoholic solution of picric acid added dropwise with stirring until further addition caused no more turbidity. After standing at 0° overnight the crude picrate was filtered off and washed with cold absolute ethanol and ether. After two recrystallizations, first from 10 ml. and then from 5 ml. of hot water, 0.656 gm. of lysine* picrate was obtained. Yield, 26 per cent.

The picrate was converted to the hydrochloride by dissolving it in 13 ml. of hot water and 2.3 ml. of concentrated HCl. After the solution was cooled, the picric acid was extracted with ether. The aqueous phase, on evaporation *in vacuo* and drying over H_2SO_4 and NaOH, gave DL-lysine dihydrochloride* in quantitative yield.

Resolution of DL-*Lysine**—The DL-lysine was resolved by the carbobenzoxy-aniline-papain method of Bergmann *et al.* (12–14).

Carbobenzoxy-DL-*Lysine**—0.430 gm. of DL-lysine* dihydrochloride was dissolved in 2.9 ml. of 2 N NaOH and cooled in ice. 1.1 ml. of carbobenzoxychloride and 2.3 ml. of 4 N NaOH were added in four portions, and the mixture shaken vigorously for 25 minutes while cooling in ice. After extraction with ether the aqueous phase was acidified with HCl and the carbobenzoxylysine taken up with ether. It weighed 0.733 gm. after evaporation and drying *in vacuo;* m.p. 100–103°.

Carbobenzoxy-L-*Lysine Anilide**—0.730 gm. of carbobenzoxy-DL-lysine* was dissolved in 1.9 ml. of N NaOH and 1.8 ml. of water. To this solution were added 0.46 ml. of aniline, 7.1 ml. of a 0.3 per cent aqueous solution of cysteine hydrochloride, 8.2 ml. of citrate buffer solution (pH 5.0), 16.4 ml. of water, 2.5 ml. of a solution of 0.100 gm. of papain in 2 ml. of water, and 2 ml. of citrate buffer. After incubation at 40° for 19 hours, the L-anilide was filtered off and washed once with 1 per cent potassium bicarbonate solution and thrice with water. Yield, 0.465 gm. Recrystallized from 50 per cent ethanol, it melted at 121–122°.

Analysis—$C_{28}H_{31}O_6N_2$ (489.55).	Calculated.	C 68.70, H 6.38, N 8.59
	Found.	" 68.65, " 6.65, " 8.75

L-*Lysine Dihydrochloride**—The anilide was refluxed with 4.5 ml. of 6 N HCl for 2 hours. After cooling, the mixture was diluted with 30 ml. of water and made alkaline with freshly prepared silver oxide. After the silver chloride and excess silver oxide were filtered off and washed with water, the filtrate was extracted with ether. The clear aqueous phase was concentrated *in vacuo* to 20 ml., acidified with HCl, and allowed to stand for 30 minutes with occasional shaking. After filtration the liquid was evaporated to dryness *in vacuo* over NaOH and H_2SO_4 to give a quantitative yield of lysine dihydrochloride. $[\alpha]_D^{23} = +15.95°$, in water; $c = 5.46$. Berg (15) reports rotations from +15.63° to +16.55° for values of c from 3.00 to 16.00.

D-*Lysine Dihydrochloride**—The filtrate (without washings) of the L-anilide was incubated with 25 mg. of papain for 5 days at 40°. After removing the precipitated mixed L- and D-anilides, the filtrate was acidified with HCl and extracted with ether. 0.243 gm. of carbobenzoxy-D-lysine remained upon evaporation of the solvent. After refluxing with 3.0 ml. of 6 N HCl for 2 hours, extraction with ether, and evaporation of the aqueous phase, 0.130 gm. of D-lysine dihydrochloride was obtained. This was recrystallized from ethanol and yielded 99 mg. of purified material. $[\alpha]_D^{23} = -13.5°$, in water; $c = 4.47$. The rotation corresponds to approximately 92.5 per cent D and 7.5 per cent L form.

Analysis—$C_6H_{14}O_2N_2 \cdot 2HCl$.	Calculated.	N 12.79, Cl 32.36
	Found.	" 12.44, " 31.82

The total average yield of L- and D-lysine dihydrochloride obtained in several complete runs was 6 per cent, calculated on the KCN used. In an experiment in which $BaCO_3$ containing 4 millicuries of radioactivity was used, 230 mg. of L-lysine dihydrochloride with a specific radioactivity of 24,000 counts (corrected) per minute per mg. were obtained.

All the radioactivity measurements are expressed as counts (corrected) per minute. They were obtained with standard sample geometry, and corrected for background and resolving time. By means of empirically determined curves of self-absorption loss in different thicknesses of sample, they were corrected to maximum specific activity (16).

DL-α-*Aminoadipic Acid*—DL-α-Aminoadipic acid was prepared by a modification of the method of Sorensen (17). 1.228 gm. of chlorobutyronitrile and 2.060 gm. of diethyl sodium phthalimidomalonate were refluxed in a bath at 160–165°. After 4 hours the alkaline reaction had disappeared, and the excess nitrile was removed by steam distillation. The residue was cooled in ice and washed with water several times by triturating and decanting. It weighed 2.050 gm. after drying. After solution in 12.5 ml. of absolute ethanol, it was heated under a reflux on a steam bath with 11 ml. of 4 N NaOH for 3 hours. 16 ml. of concentrated HCl were then added, the heating continued for 3 hours, and the solution evaporated on the steam bath. The residue was cooled in ice and extracted by repeated washing with ice-cold 33 per cent hydrochloric acid. The solution was filtered through a coarse fritted glass funnel, and evaporated. The residue, weighing 1.206 gm. after drying over H_2SO_4 and NaOH, was dissolved in 2.5 ml. of water, filtered into a 10 ml. beaker, and washed with 1.3 ml. of water. The aminoadipic acid was precipitated by titrating to pH 3.1 with 5 N NH_4OH. After standing for 4 hours at 22°, it was filtered off and washed with a few ml. of water, alcohol, and ether. Yield, 0.629 gm. (62 per cent based on diethyl sodium phthalimidomalonate).

The α-aminoadipic acid was resolved in the same manner as was lysine by the carbobenzoxy-anilide-papain method of Bergmann.

Carbobenzoxy-DL-α-aminoadipic acid, m.p. 124°.

Analysis—$C_{14}H_{17}O_6N$ (295.28). Calculated. C 57.28, H 5.82, N 4.75
Found. " 57.08, " 5.84, " 4.89

Carbobenzoxy-L-aminoadipic acid anilide, m.p. 170–171°.

Analysis—$C_{20}H_{22}O_5N_2$ (370.39). Calculated. C 64.85, H 5.99, N 7.56
Found. " 65.56, " 6.04, " 8.30

The L-α-aminoadipic acid melted at 205° with decomposition. Its specific rotation was $[\alpha]_D^{25} = +33.9°$, in 6 N HCl; $c = 5.49$.

Analysis—$C_6H_{11}O_4N$ (161.17). Calculated. C 44.72, H 6.88, N 8.70
Found. " 44.83, " 6.81, " 8.65

Procedure

Guinea pig liver was homogenized in the apparatus of Potter and Elvehjem (18) with a volume of saline solution equal to twice the weight of the liver. The composition of the saline solution was as follows: 0.123 M NaCl, 0.0128 M Na_2HPO_4, 0.005 M KCl, 0.0033 M $MgSO_4$. Unless stated otherwise the pH was 7.5.

The reaction mixture consisted of 2 ml. of homogenate, a sufficient quantity of a mixture of amino acids to provide a final concentration of 1.2 per cent, and 0.01 M α-ketoglutarate. The amino acid mixture corresponded approximately to the composition of casein. All of the lysine therein contained (10 mg. of the dihydrochloride) was labeled in the ϵ position with C^{14} (7400 counts (corrected) per mg. per minute). The final volume was 4 ml.; KOH was used for the neutralization.

The reaction mixture was incubated at 38° under oxygen for 6 hours, after which the pH was adjusted to 5.0. The mixture was then placed in a boiling water bath for 10 minutes, filtered, the coagulated protein thoroughly washed with water, and the washings added to the main filtrate. The non-protein filtrate thus obtained was concentrated *in vacuo*.

Results

A small fraction of the non-protein filtrate from an experimental run conducted at pH 7.5 was chromatographed on filter paper with phenol and *s*-collidine, and the paper treated with ninhydrin (19). Two radioactive ninhydrin spots were found, one in the position of lysine, the other of glutamic acid. The radioactivity in the latter spot excluded its being glutamic acid, as the probable mechanism for the conversion of lysine to glutamic acid entails cleavage of the radioactive ε-carbon of lysine. It seemed likely that the substance in question was α-aminoadipic acid, $C^{*}OOH \cdot (CH_2)_3 \cdot CH(NH_2) \cdot COOH$, derived from lysine.

Accordingly, α-aminoadipic acid was synthesized and chromatographed on filter paper. It gave the same chromatogram as the unknown radioactive substance.

This lead, that the radioactive substance in question might be α-aminoadipic acid, was followed. The main portion of the non-protein filtrate, from a reaction mixture to which 20 mg. of radioactive L-lysine dihydrochloride had been added, was hydrolyzed by boiling overnight with 20 per cent HCl in order to hydrolyze any peptides present. The latter step was necessary for satisfactory chromatography on Lloyd's reagent (20); it also converted any of the piperidone of α-aminoadipic acid, which might have been formed, to the straight chain.[1] After removal of the HCl by distillation, the hydrolysate was chromatographed on Lloyd's reagent. The fraction containing all the amino acids except the bases had about 5 per cent of the radioactivity originally added as lysine. It was concentrated to dryness, extracted with ether, and the residue taken up in water and decolorized by boiling with charcoal. The combined filtrate and washings were evaporated to dryness, taken up in 3 ml. of water, and treated with solid $Ba(OH)_2$ until the pH was 6.0. A small amount of radioactivity was in the precipitate; by far the major portion remained in the solution. Absolute ethanol was added to the latter to a final concentration of 95 per cent. The barium precipitate contained all the radioactivity originally in the solution. The barium was removed with sulfuric acid, the precipitation and resolution repeated three times, and after final removal of the barium the filtrate was concentrated to near dryness. A drop was chromatographed on filter paper. Four ninhydrin spots were obtained; one in the glutamic acid region was radioactive, and the other three were in the alanine, aspartic acid, and threonine regions.

---

[1] α-Aminoadipic acid like glutamic acid cyclizes. Both forms of both amino acids chromatograph alike on filter paper with phenol and *s*-collidine.

Concentrated HCl was added to the main portion of the filtrate and concentrated under a lamp until crystallization set in. About 100 mg. of crystals were collected. They gave a total of 630 counts (corrected) per minute. The mother liquor was found subsequently to have 7650 counts (corrected) per minute. The crystals gave three ninhydrin spots on the filter paper chromatogram; one in the glutamic acid region was radioactive, and the other two were in the aspartic acid and alanine regions.

The radioactive spot certainly contained α-aminoadipic acid mixed with glutamic acid. We have tried to separate α-aminoadipic and glutamic acids by chromatography on filter paper with a number of solvent mixtures; none effected a separation.

The hydrochloric acid mother liquor was evaporated to dryness and the dry residue dissolved in 0.1 N HCl. Solid $Ba(OH)_2$ was added until the solution was alkaline to phenolphthalein, and then ethanol to a concentration of 75 per cent. 351 mg. of barium salt were obtained, giving a total of 7650 counts (corrected) per minute. The barium was removed with H_2SO_4, the filtrate concentrated to dryness, and the residue dissolved in 0.1 N HCl. The presence of α-aminoadipic acid in it was determined by crystallization after adding to a portion of the solution, containing approximately 1650 counts (corrected) per minute, 100 mg. of non-radioactive α-aminoadipic acid as a carrier. The quantity of carrier was about 600 times that of the radioactive form.[2]

The solution was brought to pH 3.1 with ammonia and then concentrated slowly at room temperature under a low vacuum. The crystals which separated out were washed with a small amount of water and then ethanol, dried, and their radioactivity determined. Three recrystallizations were carried out, in the course of which 80 per cent of the carrier was left in the mother liquors. The specific activities of the crystals after each of the four crystallizations were consecutively 11.5, 13.7, 13.2, and 13.8 counts (corrected).

When 13.5 counts (corrected) are taken as the specific activity of the α-aminoadipic acid after addition of 100 mg. of carrier, 1350 counts (corrected) or 82 per cent of the radioactivity in the solution were in the α-aminoadipic acid formed from the radioactive lysine added to the reaction mixture. Constant specific activity was obtained, evidently, on the first recrystallization.

---

[2] This estimate was made as follows. The lysine dihydrochloride used had a specific activity of 7400 counts (corrected). The equivalent specific activity as α-aminoadipic acid was, therefore $(217/161) \times 7400 = 9970$ counts (corrected). A total of 1650 counts in the solution would, then, be given by 0.165 mg. of α-aminoadipic acid derived from the radioactive lysine added. Its dilution by the carrier would be $100/0.165 = 606$.

The crystals remaining after the third recrystallization gave the following data, which correspond to those of α-aminoadipic acid. M.p., 204–205° with decomposition.

Analysis—$C_6H_{11}O_4N$ (161.17). Calculated. C 44.72, H 6.88, N 8.70
Found. " 44.84, " 7.03, " 8.69

A number of experiments were carried out with boiled liver homogenate and with D-lysine obtained from the resolution of the synthesized radioactive DL-lysine.

TABLE I

α-Aminoadipic Acid Formation from Lysine

The reaction mixture contained, in a total volume of 4 ml. of saline solution, 0.66 gm. (wet weight) of homogenized guinea pig liver, 10 mg. of radioactive lysine dihydrochloride (7400 counts (corrected) per mg. per minute), 1.2 per cent (based on the final volume) of an amino acid mixture having a composition corresponding to that of casein, and 0.01 M α-ketoglutarate. The mixture was incubated at 38° under oxygen for 6 hours.

The values are total counts (corrected) of the barium salt of the dicarboxylic acid fraction.

pH	Treatment of homogenate	Isomer of lysine used	Total counts per min.
7.5	Boiled	L	0
7.5	Unboiled	"	3690
8.5	Boiled	"	0
8.5	Unboiled	"	1250
7.5	Boiled	D	0
7.5	Unboiled	"	180
8.5	Boiled	"	0
8.5	Unboiled	"	115

The results are summarized in Table I. They are expressed as counts (corrected) per minute in the dicarboxylic acid fraction obtained after chromatography on Lloyd's reagent and precipitation from 75 per cent ethanol as the barium salt. Only the reaction mixtures with L-lysine and unboiled homogenate at pH 7.5 and 8.5 were carried through to the final stages of identification by dilution with carrier and recrystallization to constant specific activity. There was too little radioactivity in the others.

The data show that more α-aminoadipic acid was formed at pH 7.5 than at pH 8.5. In preliminary experiments at pH values 7.5, 8.2, 8.5, and 9.0, the non-protein filtrates were chromatographed on filter paper, the ninhydrin spots in the glutamic acid region dissolved off, and their radioactivity measured. The highest counts were obtained at pH 7.5 and they diminished progressively with increasing pH.

The low counts (Table I) obtained when D-lysine was used may be ascribed to the 7.5 per cent of L-lysine in the D-lysine preparation.

No α-aminoadipic acid was formed when the homogenate was boiled before it was added to the reaction mixture and incubated.

A rough estimate of the rate of conversion of lysine to α-aminoadipic acid was obtained by comparing the total number of counts in the dicarboxylic acid fraction with that added as lysine. In a typical experiment at pH 7.5, 74,000 counts (corrected) were added as L-lysine dihydrochloride. 3700 counts (corrected) were found in the dicarboxylic acid fraction after 6 hours incubation with liver homogenate. In 6 hours, therefore, 5 per cent of the added lysine was converted to α-aminoadipic acid. Of the 10 mg. of lysine dihydrochloride (equivalent to 6.72 mg. of lysine) added, 0.336 mg. was converted to α-aminoadipic acid. Expressed as a Q value,[3] the rate was 0.065. This is about one-hundredth that of urea formation in liver slices or homogenates (21, 22) and about the same as that of the methylation of guanidoacetic acid by methionine (23).

This Q value is an underestimate. Some of the α-aminoadipic acid formed is converted to α-ketoadipic acid and to glutaric acid (24).

The solubilities of α-aminoadipic and of glutamic acids and their salts are so similar that the former could not be detected in analyses of the amino acid composition of proteins by any of the isolation methods in use. Nor have we been able to separate them by filter paper chromatography. The two amino acids are separated by chromatography on starch by the method of Moore and Stein (25), with a solvent consisting of 1 part of 0.1 N hydrochloric acid, 2 parts of *n*-propanol, and 1 part of *n*-butanol. Moore and Stein[4] and we have found that the α-aminoadipic acid emerges from the column considerably in advance of proline, whereas glutamic acid (with alanine) is in the effluent after proline. With this method we are now investigating whether or not α-aminoadipic acid is present in proteins.

DISCUSSION

The only previous report, of which we are aware, of α-aminoadipic acid in biological material is that of Blass and Macheboeuf (26). These authors isolated from cholera *Vibrio* two compounds whose elementary analyses, reactions on acetylation, and ultraviolet spectra corresponded to α-aminoadipic acid and hydroxy-α-aminoadipic acid. Neither compound was identified with certainty, but there is little room for doubt that they were the amino acids named. The α-aminoadipic acid amounted to 1.6 per cent and the hydroxy-α-aminoadipic acid to 1.0 per cent of the dry weight of the organism.

---

[3] Q is the change in amount of the substance in question expressed as if it were a gas in c.mm., at standard temperature and pressure, per mg. of dry weight of tissue used per hour. The dry weight of the liver used was 130 mg.; the time was 6 hours.

[4] Personal communication.

Neuberger and Sanger (3, 4) have presented evidence that before the α-amino group of L-lysine can be attacked by animal tissue enzymes the ε-amino group must be masked, preferably by acylation. They discussed some possible pathways of the degradation of lysine *in vivo*. In one of them, formation of α-aminoadipic acid is the first step. The latter surmise is now substantiated by the evidence presented above. α-Aminoadipic acid, as the first (or one of the first) intermediate in the degradation of lysine, is in accord with the enzymatic findings of Neuberger and Sanger, in that conversion of the ε-amino group to a carboxyl group is analogous to acylation. It also accounts for the failure of the α-amino nitrogen of lysine to participate in reversible transamination reactions *in vivo*. It is converted to α-aminoadipic acid before it yields its α-amino nitrogen.

We have previously reported (27) evidence of the probable formation of α-aminoadipic acid from lysine in kidney.

Mitchell and Houlahan (28) have found that α-aminoadipic acid can replace L-lysine in one lysine-requiring *Neurospora* mutant. The accumulation of large quantities of α-aminoadipic acid and of hydroxy-α-aminoadipic acid in cholera *Vibrio* points to unusual features of lysine metabolism in that organism which is analogous to those found in mutants of microorganisms.

SUMMARY

1. The synthesis and resolution of lysine labeled with C^{14} in the ε position and the synthesis and resolution of α-aminoadipic acid are described.

2. α-Aminoadipic acid is formed from L-lysine in guinea pig liver homogenate. D-Lysine is inactive.

3. Over the pH range 7.5 to 9.0, the reaction is fastest at pH 7.5. Boiling destroys the catalytic activity of the homogenate.

4. α-Aminoadipic acid can be separated from glutamic acid by chromatography on starch.

The authors were assisted by A. A. Dvorsky, D. Eggarter, H. E. Jeffery, G. Oppenheimer, and A. Tollestrup.

BIBLIOGRAPHY

1. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *J. Biol. Chem.*, **173**, 423 (1948).

2. Foster, G. L., Rittenberg, D., and Schoenheimer, R., *J. Biol. Chem.*, **125,** 13 (1938). Weissman, N., and Schoenheimer, R., *J. Biol. Chem.*, **140,** 779 (1941).
3. Neuberger, A., and Sanger, F., *Biochem. J.*, **37,** 515 (1943).
4. Neuberger, A., and Sanger, F., *Biochem. J.*, **38,** 119, 125 (1944).
5. McGinty, D. A., Lewis, H. B., and Marvel, C. S., *J. Biol. Chem.*, **62,** 75 (1924–25).
6. Gordon, W. G., *J. Biol. Chem.*, **127,** 487 (1939).
7. Cramer, R. D., and Kistiakowsky, G. B., *J. Biol. Chem.*, **137,** 549 (1941).
8. Loftfield, R. B., U. S. Atomic Energy Commission, Isotopes branch circular C-3.
9. Frantz, I. D., Jr., Loftfield, R. B., and Miller, W. W., *Science*, **106,** 544 (1947).
10. Organic syntheses, New York, coll. **1,** 2nd edition, 156 (1941).
11. Fischer, E., and Weigert, F., *Ber. chem. Ges.*, **35,** 3772 (1902).
12. Bergmann, M., and Fraenkel-Conrat, H., *J. Biol. Chem.*, **119,** 707 (1937).
13. Fruton, J. S., Irving, G. W., Jr., and Bergmann, M., *J. Biol. Chem.*, **133,** 703 (1940).
14. Behrens, O. K., Doherty, D. G., and Bergmann, M., *J. Biol. Chem.*, **136,** 61 (1940).
15. Berg, C. P., *J. Biol. Chem.*, **115,** 9 (1936).
16. Kamen, M. D., Radioactive tracers in biology, New York (1947).
17. Sørensen, S. P. L., *Compt.-rend. trav. Lab. Carlsberg*, **6,** 1 (1903–06).
18. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114,** 495 (1936).
19. Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, **38,** 224 (1944).
20. Bergdoll, M. S., and Doty, D. M., *Ind. and Eng. Chem.*, **18,** 600 (1946).
21. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210,** 33 (1932).
22. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **169,** 461 (1947).
23. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **132,** 559 (1940); **160,** 635 (1945).
24. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *J. Biol. Chem.*, **176,** 1395 (1948).
25. Moore, S., and Stein, W. H., *Federation Proc.*, **7,** 174 (1948). Stein, W. H., and Moore, S., *Federation Proc.*, **7,** 192 (1948).
26. Blass, J., and Macheboeuf, M., *Helv. chim. acta*, **29,** 1315 (1946).
27. Dubnoff, J. W., and Borsook, H., *J. Biol. Chem.*, **173,** 425 (1948).
28. Mitchell, H. K., and Houlahan, M. B., *J. Biol. Chem.*, **174,** 883 (1948).

THE DEGRADATION OF α-AMINOADIPIC ACID IN GUINEA PIG LIVER HOMOGENATE*

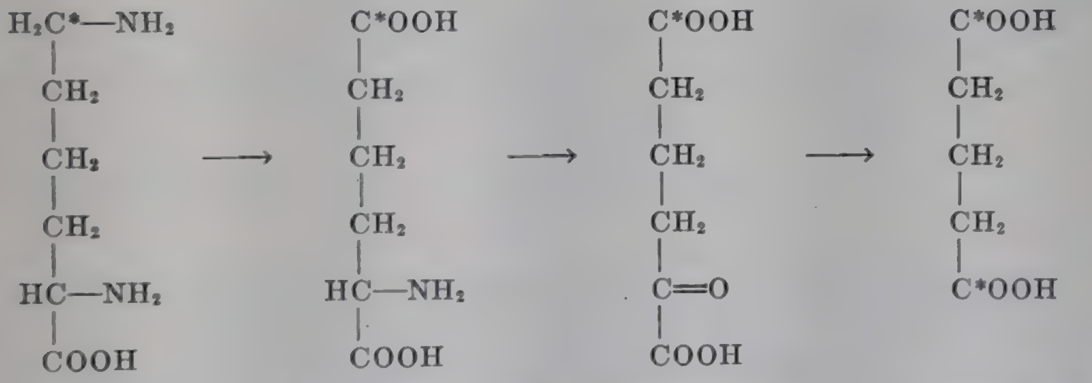
By HENRY BORSOOK, CLARA L. DEASY, A. J. HAAGEN-SMIT, GEOFFREY KEIGHLEY, and PETER H. LOWY

(From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena)

(Received for publication, June 21, 1948)

In continuation of our study of the metabolism of L-lysine, α-aminoadipic acid, which is formed from lysine in guinea pig liver homogenate (1), was synthesized with C^{14} in the ϵ-position. The metabolism of the latter compound was followed by search for the radioactive tracer among the probable metabolic products. Two have been identified, α-ketoadipic and glutaric acids.

The accompanying diagram (I) indicates one of the pathways of the catabolism of lysine. The asterisk indicates the position of the labeled carbon.



Lysine α-Aminoadipic acid α-Ketoadipic acid Glutaric acid

(I)

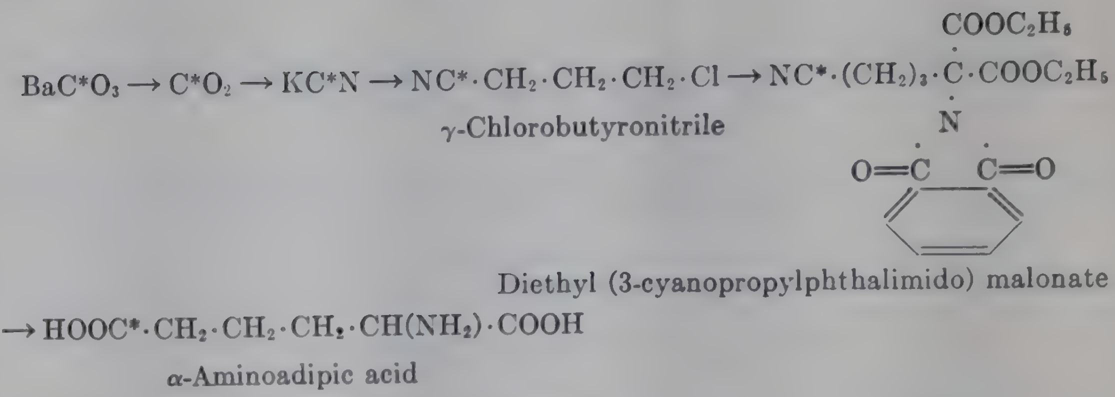
Preparations

A summary of the synthesis and resolution of α-aminoadipic acid labeled with C^{14} in the ϵ position is shown in diagram (II). The radioactive carbons are marked with an asterisk.

---

* This work is part of that done under contract with the Office of Naval Research, United States Navy Department. It was reported at the meeting of the American Society of Biological Chemists, March 15-19, 1948.

The C^{14} used in this investigation was supplied by the Monsanto Chemical Company, Clinton Laboratories, Oak Ridge, Tennessee, and obtained on allocation from the United States Atomic Energy Commission.



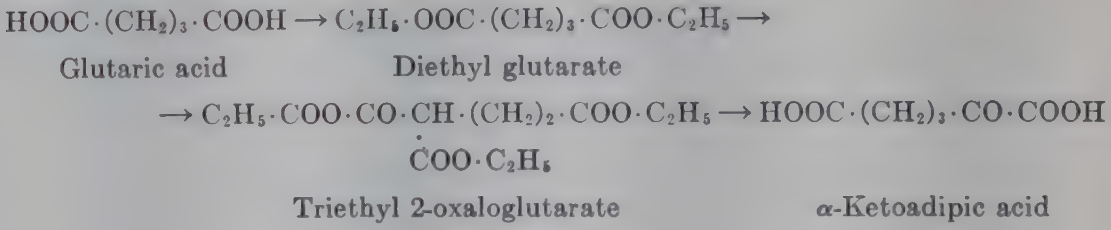
$$BaC^*O_3 \rightarrow C^*O_2 \rightarrow KC^*N \rightarrow NC^*\cdot CH_2\cdot CH_2\cdot CH_2\cdot Cl \rightarrow NC^*\cdot(CH_2)_3\cdot \overset{COOC_2H_5}{C}\cdot COOC_2H_5$$
$$\rightarrow HOOC^*\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot COOH$$

(II)

The details of the preparation have been described in a previous communication (1).

89.3 mg. of $BaC^{14}O_3$ containing 0.625 millicurie of C^{14} yielded 0.127 gm. of pure L-α-aminoadipic acid, giving 14,450 counts (corrected) per mg. per minute.

α-Ketoadipic Acid—Diagram (III) is a summary of the synthesis of α-ketoadipic acid.



$$HOOC\cdot(CH_2)_3\cdot COOH \rightarrow C_2H_5\cdot OOC\cdot(CH_2)_3\cdot COO\cdot C_2H_5 \rightarrow$$
$$\rightarrow C_2H_5\cdot COO\cdot CO\cdot \underset{COO\cdot C_2H_5}{CH}\cdot(CH_2)_2\cdot COO\cdot C_2H_5 \rightarrow HOOC\cdot(CH_2)_3\cdot CO\cdot COOH$$

(III)

Diethyl glutarate was prepared by the method of Locquin and Elghozy (2), and triethyl 2-oxaloglutarate by the method of Gault (3). The latter compound was decarboxylated with HCl. After removal of the HCl *in vacuo* spontaneous crystallization set in. The crude product was purified by solution in warm ether and reprecipitation by the addition of an equal volume of petroleum ether. The light orange crystals had the following composition.

$C_6H_8O_5$ (160.06). M.p. 124°. Calculated, C 44.98, H 5.04; Found, C 45.12, H 5.04

Procedure

The preparation of the guinea pig liver homogenate and the saline solution are described in a previous communication (1).

In a representative experiment 2 ml. of homogenate containing 0.66 gm. of liver (wet weight) and 2 ml. of saline solution containing a mixture of 5 mg. of L-α-aminoadipic acid (14,450) counts (corrected) per minute per mg.) and 5 mg. of non-radioactive L-α-aminoadipic acid were added to each of four 20 ml. beakers. Two were immediately brought to pH 5.0 and boiled for 10 minutes, the coagulated protein extracted with water, and the non-protein filtrates and washings combined. The other two beakers were incubated at pH 7.4 under oxygen at 38° for 6 hours, then deproteinized by boiling at pH 5.0, and their non-protein filtrates combined.

Results

The non-protein filtrates were cleared by boiling with charcoal, acidified with hydrochloric acid to a concentration of 0.1 N, and then extracted with ether. After the ether was evaporated, the residue was taken up in 3 ml. of water; 100 mg. of non-radioactive α-ketoadipic acid and 91 mg. of phenylhydrazine hydrochloride dissolved in 2 ml. of water were added. The oil, α-ketoadipic acid phenylhydrazone, which settled out first, crystallized in 2 hours. The recrystallization procedure was as follows: 2 ml. of water were added to the crystals, and the suspension brought to boil and then treated with ethanol dropwise until all the crystals dissolved. The solution was then cooled, the crystals collected, and their specific radioactivity determined.

TABLE I

Specific Radioactivity of α-Ketoadipic Acid Phenylhydrazone after Successive Recrystallizations

No. of crystallizations	Counts (corrected) per mg. per min. of phenylhydrazone from	
	Reaction mixture at zero time	Reaction mixture after 6 hrs. incubation
1	1.8	10.1
2	1.0	3.1
3	0	2.7
4	0	2.6
5	0	2.7
6	0	2.8

Table I gives the specific radioactivities of the phenylhydrazone samples obtained from the reaction mixtures at zero time and after 6 hours incubation. The figures show that by the second recrystallization all the radioactivity had been removed from the phenylhydrazone obtained from the zero time reaction mixture, and that constant specific activity had been attained in the case of the phenylhydrazone obtained after 6 hours incubation. The counts in the first and second crystallizations probably arose from α-aminoadipic acid, and, in the case of the 6 hour reaction mixture, also from glutaric acid arising from degradation of the α-ketoadipic acid formed from the added α-aminoadipic acid.

The melting point of the radioactive phenylhydrazone after the fifth crystallization was 141–142°, and of a mixture with an authentic sample 141–142°. Gault (3) reported the melting point as 141°. After the final recrystallization, 15 mg. of the phenylhydrazone remained. 90 per cent of the added α-ketoadipic acid was, therefore, left in the mother liquors.

These data prove that the α-aminoadipic acid was oxidatively deaminized to α-ketoadipic acid.

The rate of formation of α-ketoadipic acid from α-aminoadipic acid can be calculated from the value of the constant specific activity of the α-ketoadipic acid phenylhydrazone obtained from the reaction mixture incubated for 6 hours. The specific activity of the phenylhydrazone was 2.7 counts (corrected) per mg. per minute. 100 mg. of α-ketoadipic acid, equivalent to 156.3 mg. of its phenylhydrazone, were added as a carrier. The weight of the radioactive form being neglected, the total α-ketoadipic acid in the solution contained 156.3 × 2.7 = 422 counts (corrected). 144,500 counts (corrected) were added originally as 20 mg. of α-aminoadipic acid. 0.29 per cent of the latter was found as the keto acid, or 0.058 mg. Expressed as a Q value,[1] this rate is 0.005. It is about one-twelfth that of the formation of α-aminoadipic acid from L-lysine (1).

TABLE II

Specific Radioacitivity of Barium Glutarate (Dried at 100°) after Successive Reprecipitations

No. of precipitations	Counts (corrected) per mg. per min. of barium glutarate from	
	Reaction mixture at zero time	Reaction mixture after 6 hrs. incubation
1	0	1.50
2	0	1.33
3		1.30
4		0.95
5		1.01
6		1.01

In another experiment similar to the preceding, the non-protein filtrate was examined for evidence of formation of glutaric acid. The procedure was the same as before to the stage after evaporation of the ethereal extract of the non-protein filtrate. 100 mg. of non-radioactive glutaric acid were added as a carrier in the subsequent crystallization. The mixture was taken up in 3 ml. of water and treated with saturated barium hydroxide solution to pH 9.0; the final volume was 5 ml. No precipitation occurred. 10 ml. of 95 per cent ethanol were then added. The precipitate of barium glutarate pentahydrate was filtered off. 96 per cent of the water of hydration was driven off by drying at 100°, and the specific radioactivity of the compound was determined.

---

[1] Q is the rate of change of the substance in question expressed as if it were a gas in c.mm., at standard temperature and pressure, per mg. of dry weight of tissue used per hour.

The solution in water and precipitation with ethanol were repeated five times. Table II gives the specific radioactivities of the barium glutarate samples obtained as above from the reaction mixtures at zero time and after 6 hours incubation. After the final reprecipitation 30 mg. of barium salt (as pentahydrate) remained, corresponding to 11 per cent of the original glutaric acid added.

Analysis of the radioactive barium salt after the last precipitation gave, on an anhydrous basis, 51.3 per cent of barium (calculated, 51.4 per cent).

These data prove that glutaric acid was one of the products formed in the liver homogenate from the added α-aminoadipic acid. There can be little doubt that α-ketoadipic acid was formed first, and that the glutaric acid arose by its oxidative decarboxylation.

The calculation of the rate of formation of glutaric acid from the added α-aminoadipic acid is as follows: the final constant specific activity of the barium glutarate was 1.0 count (corrected) per mg. per minute; 100 mg. of glutaric acid, equivalent to 202.5 mg. of anhydrous barium glutarate, were added as carrier; the glutaric acid formed from the added α-aminoadipic acid contained, therefore, 202 counts (corrected) per minute; 144,500 counts were contained in the α-aminoadipic acid; 0.14 per cent of the latter was, therefore, found as glutaric acid. The Q value is 0.0024.

Of the two successive oxidative steps in the degradation of α-aminoadipic acid, deamination followed by decarboxylation, the latter is probably the faster. 0.43 per cent of the added α-aminoadipic acid was found in the above two degradation products. This figure represents the rate of its deamination. The corresponding rate of decarboxylation was 0.14/0.43 or 33 per cent.

SUMMARY

1. The synthesis of α-ketoadipic acid is described.

2. In guinea pig liver homogenate α-aminoadipic acid is oxidatively deaminized to α-ketoadipic acid and the latter is oxidatively decarboxylated to glutaric acid.

3. The deamination of α-aminoadipic acid is much slower than its formation from L-lysine. The decarboxylation of α-ketoadipic acid is faster than the deamination of α-aminoadipic acid.

4. The foregoing evidence was obtained with the use of lysine and of α-aminoadipic acid labeled with C^{14} in the ε position.

The elementary analyses were carried out by Dr. G. Oppenheimer. The authors were assisted in this work by A. A. Dvorsky, D. Eggarter, H. E. Jeffery, and A. Tollestrup.

BIBLIOGRAPHY

1. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G, and Lowy, P. H., *J. Biol. Chem.*, **173**, 423 (1948); **176**, 1383 (1948).
2. Locquin, R., and Elghozy, F., *Bull. Soc. chim.*, **41**, 445 (1927).
3. Gault, H., *Bull. Soc. chim.*, **11**, 382 (1912).

A STUDY OF CONDITIONS FOR KJELDAHL DETERMINATION OF NITROGEN IN PROTEINS

DESCRIPTION OF METHODS WITH MERCURY AS CATALYST, AND TITRIMETRIC AND GASOMETRIC MEASUREMENTS OF THE AMMONIA FORMED

By ALMA HILLER, JOHN PLAZIN, AND DONALD D. VAN SLYKE

(From the Hospital of The Rockefeller Institute for Medical Research, New York)

(Received for publication, July 15, 1948)

The publications on Kjeldahl's method for determining nitrogen that have appeared since Kjeldahl's (1) original paper in 1883 perhaps outnumber those on any other analytical method in the same period of time.[1] The diversity of papers is attributable to the immense usefulness of the method, to its need for modifications for applications to various types of organic and inorganic compounds, and to the search for catalysts to provide such modifications and to accelerate the digestion. Kjeldahl himself used a digestion mixture of sulfuric and phosphoric acids which he found was adequate for many organic substances. For alkaloids, however, he found the addition of an oxidizing agent necessary and added potassium permanganate crystals to the hot concentrated digest.

The substances that have been added to the sulfuric acid digest may be divided into three classes: (1) Potassium sulfate to increase the boiling point and thereby accelerate the digestion process. (2) Oxidizing agents. The requirement for these is that they must assist the digestion of organic compounds without destroying any of the ammonia formed. Permanganate was thus used by Kjeldahl. At present the oxidizing agents in most general use are hydrogen peroxide and potassium persulfate. (3) Metallic catalysts and other substances that act as accelerators. Phosphoric acid employed by Kjeldahl is such an accelerator; although its boiling point is lower than that of concentrated sulfuric acid, the presence of phosphoric acid makes digestion go faster. Phosphoric acid has the disadvantage of etching the digestion flask. The metallic catalysts used to accelerate digestion include mercury, copper, and selenium.

---

[1] The early history of the Kjeldahl procedure from 1883 to 1892 has been fully presented by Vickery (2), and reviews by Friedrich (3) in 1933 and Bradstreet (4) in 1940 give bibliographies.

Potassium sulfate to accelerate the digestion by raising the boiling point was introduced by Gunning (5) in 1889. Mercury was introduced by Wilfarth (6) in 1885. Arnold (7) in 1886 used sulfuric acid with both copper and mercury as catalysts. The Gunning-Arnold procedure was thus named by Arnold and Wedemeyer (8) who recommended a digestion mixture containing both the potassium sulfate introduced by Gunning and the copper and mercury introduced by Arnold. In this country the so called Gunning-Arnold method used as a standard macro-Kjeldahl procedure for several decades has employed a mixture of sulfuric acid, copper sulfate, and potassium sulfate, with or without mercury.

In testing the various modifications of the Kjeldahl method for pure organic substances the results have commonly been compared with the theoretical nitrogen contents of the substances or with the values obtained by the classic Dumas combustion. In applying the various Kjeldahl procedures to protein material, however, theoretical nitrogen values are not available and in most instances Dumas combustions have not been used for comparison, the criterion for satisfactory results being comparison with maximum nitrogen values obtained by other Kjeldahl procedures taken as standards.

The work in the present paper grew out of the observation that when dried preparations of plasma proteins were analyzed for nitrogen by the Campbell-Hanna (9) Kjeldahl procedure, in which a mixture of sulfuric and phosphoric acids with copper and selenium as catalysts is employed and digestion is continued for 12 minutes after clearing, results were several per cent lower than by the Dumas dry combustion. A survey of various digestion mixtures, including metallic catalysts, persulfate, and peroxide, resulted in the conclusion that none of the catalysts that have hitherto been used except mercury would give within a reasonable period of time a nitrogen yield from proteins equal to that of the Dumas combustion.

Presumably the reason why mercury has been little used in routine macro- or micro-Kjeldahl procedures in current use is the added step in the analysis made necessary by the fact that when alkali is added to a mercury-containing solution of ammonia, as a preliminary to distillation of the ammonia, a considerable fraction of the ammonia is bound by the mercuric oxide precipitated and cannot be liberated by boiling. To prevent low results from this cause it has been customary to add either a sulfide, or thiosulfate, which liberates sulfide, in order to precipitate the mercury. The addition of sulfide to the acid solution of digest causes an unpleasant evolution of hydrogen sulfide, and the heavy precipitate of mercuric sulfide increases the tendency to bumping during digestion. These unpleasant features can be avoided by the use of zinc dust to reduce the mercuric oxide to metallic mercury during the distillation. This device was introduced in 1892 by Böttcher (10) and was used by Arnold and Wedemeyer (8), but apparently has since been overlooked. The zinc dust not only reduces the mercuric oxide to metallic mercury but also, by providing an evolution of fine bubbles of hydrogen gas, causes the boiling during digestion to proceed very smoothly and with minimum danger of bumping.

Sörensen and Andersen (11) studied the application of the Kjeldahl procedure to lysine and found that addition of mercury to the digest was necessary for accurate results. They concluded that any Kjeldahl procedure applied to proteins or their derivatives should be checked by comparison with the Gunning-Arnold method. Miller and Houghton (12) confirmed the necessity of mercury in analysis of lysine. Pregl and Roth (13) recommended the use of mercury in analyses of protein material, but did not emphasize the necessity of the addition.

In the present paper details of a procedure are given for use of a mercury-containing digest with three types of Kjeldahl analyses: (1) macroanalyses, (2) microanalyses with distillation and titration of the ammonia, and (3) microanalyses with gasometric determination of the ammonia by the hypobromite reaction.[2]

For determination of the ammonia in the distillate in the macromethod we have found the procedure of Meeker and Wagner (14) advantageous, compared with the older method of collecting the distilled ammonia in a solution of excess standard acid and titrating back the excess with standard alkali. In the Meeker and Wagner procedure the ammonia is distilled into boric acid solution and is titrated by adding standard sulfuric acid to the distillate until the pH is lowered to that of a control standard with boric acid alone. This procedure eliminates the necessity of using standard alkali. For titrations in the micromethod, however, we have found that routine analyses are more accurate when the older procedure of distilling into excess standard acid is employed, with back titration with alkali. For the small amounts of ammonia present in the microanalyses this procedure gives a sharper end-point than the titration in the presence of boric acid. In the gasometric micromethod the hypobromite procedure for ammonia described by Van Slyke and Kugel (15) is employed, the mercury being first removed by boiling for a moment with zinc dust.

[2] A photometric microprocedure, in which the same digestion mixture is used, has been employed by Dr. Howard Eder in this laboratory and will be reported by him later. After digestion the acid digest is diluted and treated with a small amount of Na_2S. The excess H_2S is removed by boiling for a minute or two, and the ammonia is determined by nesslerizing an aliquot of the clear supernatant.

METHODS

Macro-Kjeldahl

Special Apparatus Required

A still with *glass* condenser tubes.[3]

Glass spoons to measure 10 gm. of K_2SO_4 and 2 gm. of zinc dust. These spoons are of the type used by Van Slyke and Folch ((17) Fig. 4) and described in detail by Van Slyke, Hiller, Weisiger, and Cruz (18).

Reagents

Powdered potassium sulfate, ammonia-free.

Mercuric sulfate solution. Dilute 12 ml. of concentrated H_2SO_4 to 100 ml. with water, and dissolve 10 gm. of red mercuric oxide in this solution.

Concentrated sulfuric acid, C.P.

Zinc dust (not granulated zinc), ammonia-free.

Concentrated sodium hydroxide solution, approximately 18 N.

Boric acid, 4 per cent solution.

Sulfuric acid, N/14 solution.

Indicator. The mixed indicator described by Meeker and Wagner (14) or a 0.1 per cent solution of brom-cresol green in 95 per cent ethyl alcohol.

Procedure

Into 500 ml. Kjeldahl flasks measure the samples to be analyzed, add to each 10 gm. of potassium sulfate, 10 ml. of the mercuric sulfate solution, and 20 ml. of concentrated sulfuric acid. Digest over a low flame until frothing ceases and water has been driven off, then with "subboiling" (*i.e.*, heating just under the boiling point, so that there is an occasional slight ebullition, as originally recommended by Kjeldahl (1)), which is continued for 2 hours after clearing. Cool, add 250 ml. of water, and after cooling add 2 gm. of zinc dust and 50 ml. of 18 N NaOH. Distil into 500 ml. receivers, each containing 50 ml. of 4 per cent boric acid. After 10 or 15 minutes distillation all the mercuric oxide is reduced to metallic mercury, which amalgamates with the zinc, and the solutions become clear. Distillation is continued until about 200 ml. of distillate are collected, or slight bumping begins.

For titration of the ammonia in the distillate add either 5 drops of the mixed indicator of Meeker and Wagner (14) or 0.3 ml. of 0.1 per cent alcoholic brom-cresol green solution and titrate with the N/14 sulfuric acid till the color matches that of a control flask. The control is prepared by measuring into a flask of the type used as receiver 50 ml. of boric acid, indicator solution, and enough water to make the volume equal to that in the receiver containing the distillate.

---

[3] Some of the metallic mercury formed by action of the zinc distils into the condensers, and, if the condensing tubes are of block tin, they are eventually destroyed by amalgamation with the mercury. The importance of using glass condensers in place of the usual metal condensers in Kjeldahl stills, when mercury is in metallic form during distillation, has been pointed out by Andersen and Jensen (16) in their very thorough paper on the Kjeldahl method.

Blank analyses are performed with all the reagents used.

Calculation

$$T - B = \text{mg. N in sample analyzed}$$

T indicates the ml. of N/14 H_2SO_4 used in titration of the distillate, B the ml. used in titrating the blank.

Titrimetric Micro-Kjeldahl

Apparatus

Glass spoons to measure 0.5 gm. of K_2SO_4 and 0.2 gm. of zinc dust ((17) Fig. 4; (18)).

Funnel to deliver K_2SO_4 into the bottom of the digestion tube. This should have a stem 12 cm. long and 1 cm. in diameter. It can be made from glass tubing by flaring one end into a funnel.

Test-tubes of Pyrex glass, 22 to 25 by 200 mm. for digestion.

Erlenmeyer flasks of 125 ml. capacity, to serve as receivers.

Burette, 10 ml. calibrated.

Calibrated pipettes, 1, 2, 5, and 10 ml.

Micro digestion rack.

Apparatus for steam distillation of ammonia from the digests.[4]

Reagents

The *K_2SO_4, concentrated H_2SO_4, mercuric sulfate solution*, and *zinc dust*, described for the macroanalysis.

---

[4] A convenient micro steam distillation apparatus is the type described by Parnas and Wagner (13, 19) in which the contents of the distilling flask are removed automatically by suction at the end of each distillation, and in which no burner is used under the distilling flask. The distilling flask devised by Parnas and Wagner (13, 19) as described in the catalogues of the laboratory supply companies is good, except that in the round bottomed distilling flask the end of the bent inner tube is placed about 0.5 cm. too high to remove the solid particles by suction when zinc dust is used. The end of the inner tube should approach that section of the rounded portion of the flask which is the *bottom when placed at the angle used for distillation*. The ideal flask for use with zinc dust is one in which the bottom is cone-shaped, with a straight (unbent) steam inlet ending within a few mm. of the bottom. Distilling flasks having these requirements for use with zinc dust were supplied by E. Machlett and Son, 220 East 23rd Street, New York. The condenser should be of glass, not metal, when mercuric sulfate is used as catalyst.

Alundum chips, black (Norton's No. 14).

Sodium hydroxide, approximately 10 N solution. Dissolve 400 gm. of NaOH in water and dilute to 1 liter.

Standard 71.4 mM ammonium chloride solution, to be used for checking the micro-Kjeldahl procedure. Dissolve 0.382 gm. of NH_4Cl, sublimed "analytical reagent" grade, in water, and dilute to 100 ml. 1 ml. contains 1 mg. of nitrogen.

Acetate buffer with pH 5, 0.2 M. To 1 liter of 0.2 M sodium acetate (27.22 gm. of $NaC_2H_3O_2 \cdot 3H_2O$ per liter) add 427 ml. of 0.2 N acetic acid (standardized by titration against 0.1 N NaOH with phenolphthalein indicator).

Alizarin red indicator solution, 0.1 per cent in water.

Control flask for titration end-point. Measure into a 125 ml. Erlenmeyer flask 7 ml. of the 0.2 M acetate buffer, 63 ml. of distilled water, and 0.8 ml. of 0.1 per cent alizarin red indicator solution. This control flask should be made up fresh at least every 3 days, or oftener if molds grow in the solution.

0.01428 N (N/70) H_2SO_4, made by dilution of standard 1 N or 0.1 N H_2SO_4 (14.28 or 142.8 ml. respectively to 1 liter).

0.01428 (N/70) NaOH solution. The solution is kept in a heavily paraffined bottle protected against atmospheric CO_2 by a soda lime tube. The solution is standardized *daily* by titration against 10 ml. portions of the 0.01428 N H_2SO_4 with the same pH and volume at the end-point described below for titration of distilled ammonia.

Procedure for Titrimetric Microanalysis

Digestion—Into Pyrex glass tubes 22 to 25 by 200 mm. measure 0.5 gm. of K_2SO_4. (For the determination of albumin in a filtrate when the globulin has been precipitated with sodium sulfate, no K_2SO_4 need be added.) Samples containing 0.2 to 2 mg. of nitrogen are added, followed by 0.5 ml. of the mercuric sulfate solution, 1 ml. of concentrated sulfuric acid, and three or four pieces of alundum. The mixture is boiled gently over a micro burner until the water is boiled off; then the flame is increased slightly so that the concentrated digest is constantly boiling with *slight* motion. When entirely clear, gentle boiling is continued for 30 minutes. As soon as the tubes are sufficiently cool, about 2 minutes, but *before* the contents solidify, wash down the sides of the tubes with 3 ml. of water.

Distillation—Each distilling apparatus should be tested for the volume of distillate required under the conditions used for distillation, to give theoretical results with a standard ammonium chloride solution.

Before each series of distillations the distilling apparatus is steamed out for 30 minutes. Before each digest is transferred from the digestion tube to the distilling flask, the lip of the digestion tube is greased lightly with a little vaseline to avoid loss during transfer and washing. The contents of the tube are washed into the distilling flask with four portions of 1.5 to 2 ml. each of water. The sides of the tube are rinsed down with each portion. While the third washing is in the funnel of the Parnas distilling apparatus, add to it, in the funnel, 0.2 gm. of zinc dust. Deliver the mixture of washing and zinc dust into the distilling flask and follow it with the fourth washing. Deliver 5 ml. of 10 N NaOH into the distilling flask and distil into 10 ml. of 0.01428 N H_2SO_4 with the tip of the condenser below the surface of the acid. Distil a volume found to be required by the test with standard ammonium chloride solution as described above, then lower the receiving flask so that the tip of the condenser is above the surface of the acid, and distil 1 minute longer. Wash the tip of the condenser into the flask with a few drops of water.

Titration—To each distillate add 0.8 ml. of 0.1 per cent alizarin red indicator solution and titrate with 0.01428 N NaOH from a 10 ml. burette till the color matches that of the acetate buffer solution in the control flask. The final volume should approximate that in the control flask.

Blank analyses should be done frequently; the entire procedure should be followed through.

Calculation

$$\text{Mg. N in sample analyzed} = 0.2\ (B - T)$$

B = ml. of 0.01428 N NaOH required in the back titration of blank analyses;
T = ml. of 0.01428 N NaOH required in the titration of the distillate.

Gasometric Micro-Kjeldahl

Apparatus

The Van Slyke-Neill manometric apparatus (20, 21).

The *glass spoons, funnel,* and *Pyrex digestion tubes* described for the titrimetric microanalysis.

Reagents

The *K_2SO_4, concentrated H_2SO_4, mercuric sulfate solution,* and *zinc dust,* described for the macroanalysis.

Alundum chips, black (Norton's No. 14).

Hydrochloric acid, approximately 0.01 N.

Sulfuric acid, approximately 3.5 N (100 ml. of concentrated H_2SO_4 diluted to 1 liter with water).

Brom-thymol blue, 0.4 per cent.

Sodium hydroxide, approximately 10 N.

Bromine solution. 60 gm. of KBr are dissolved in 100 ml. of water. 2.5 ml. of bromine are dissolved in the KBr solution.

Procedure

Digestion—Digestion is carried out as described for the micro titrimetric procedure.

Treatment of Digest—After cooling the digest for 2 minutes, 3 ml. of water and 0.2 gm. of zinc dust are added, and the contents of the digestion tube are heated till they begin to boil, to accelerate amalgamation of zinc and mercury. The mixture is then cooled, a drop of 0.4 per cent bromthymol blue is added, and the solution is neutralized, as described by Van Slyke (22), by adding from a pipette 10 N sodium hydroxide, a drop at a time, with occasional cooling of the tube in cold water. Addition of alkali is continued until the mixture becomes alkaline to the indicator. 3.5 N sulfuric acid is then dropped in until the color just changes back to acid, in order to prevent loss of ammonia during subsequent deaeration.

Transfer of Digest to Van Slyke-Neill Chamber—The digest solution, after the above treatment, is decanted into the cup of the Van Slyke-Neill blood gas apparatus and the volume is noted. The solution is then drawn down into the chamber. Into a graduated pipette is drawn enough 0.01 N HCl to make the total volume of the solution up to 10 or 11 ml., and this HCl is used in three portions to rinse into the gas apparatus the drops of solution adherent to the walls of the digestion tube and to the zinc-mercury amalgam in the bottom of the tube. Each portion is used to rinse the walls of the digestion tube, then the walls of the cup of the gas apparatus, and is then drawn down into the chamber.

Gasometric Determination of Ammonia—The cock of the gas chamber is sealed with a drop of mercury, the chamber is evacuated, and is shaken 2 minutes to extract the air from the solution. The extracted air bubble is ejected. The extraction is repeated, and the slight air bubble obtained is ejected.[5] Then 1.25 ml. of 10 N NaOH are placed in the cup of the chamber, and 0.75 ml. of the bromine solution is added and mixed with the alkali. 1.5 ml. of the mixture are run into the chamber, and the cock is sealed with mercury. The chamber is then evacuated and shaken 3 minutes.

Measurement of Evolved N_2—The gas volume is reduced to 2 ml. and the reading, p_1 mm., on the manometer is noted, together with the temperature. The gas is then ejected from the chamber, the meniscus of the solution is lowered to the 2 ml. mark, and p_2 is read on the manometer.

---

[5] If the 10 N NaOH used to neutralize the digest has been permitted to absorb a large amount of atmospheric CO_2, the gas extracted from the digest solution at this point will contain more CO_2 than air, and the second extraction will yield a relatively large bubble, almost entirely CO_2. A large bubble on the second extraction does not indicate the necessity for a third extraction; the two extractions remove the air completely, and any CO_2 that remains in the solution does not contaminate the N_2 evolved by the hypobromite reaction, since the latter is carried out in strongly alkaline solution.

Blank determinations are performed with the entire procedure.

Calculations are made as described by Van Slyke (22) from the table of factors on p. 242 of his paper (22).

EXPERIMENTAL

Preparation and Analysis of Total Protein Prepared from Plasma

Total proteins were prepared from two different lots of Lyovac (lyophilized pooled normal human plasma of Sharp and Dohme) and from fresh dog plasma. Of the Lyovac, 2 gm. were dissolved in 50 ml. of water. Dog plasma was obtained from freshly drawn dog blood, with potassium oxalate as anticoagulant. Since the nitrogen values obtained are used to estimate the factor by which the nitrogen of normal human and dog plasma proteins is multiplied to calculate the protein, the purification of the proteins is outlined in some detail.

Lipides were removed by treatment with alcohol and ether. To 50 ml. of the Lyovac solution, or to 20 ml. of dog plasma to which 30 ml. of water had been added, 225 ml. of absolute alcohol, then 225 ml. of ethyl ether were added with shaking. After mixing well, the mixture was allowed to stand for 2 hours until the supernatant fluid was clear. About 300 ml. of the clear supernatant fluid were siphoned off. The precipitate, with the remainder of the supernatant, was transferred to a 250 ml. centrifuge bottle, centrifuged, and the supernatant fluid decanted off. The residue was washed once with 200 ml. of a mixture of 1 part of water to 4.5 parts each of absolute alcohol and ether. After thorough mixing with a footed rod, it was centrifuged and decanted. The residue was washed twice with 200 ml. portions of anhydrous ether in the same manner. The last traces of ether were removed from the residue in the centrifuge bottle by running a gentle current of air over the surface.

Mineral salts were removed by the procedure described by Robinson and Hogden (23). The finely powdered residue was completely dissolved in 200 ml. of water, the pH adjusted to 5.0 to 5.2 with 0.1 N acetic acid, and the solution placed in a boiling water bath for 1 hour. After cooling to room temperature, the coagulum was centrifuged, and the supernatant solution was removed by decantation. The residue was washed eight times with 150 ml. portions of boiling water, twice with 150 ml. portions of absolute alcohol, and three times with 150 ml. portions of anhydrous ether. The last traces of ether were removed from the residue by a gentle current of air.

The finely powdered residue was spread on hardened filter paper on a large watch-glass and air-dried by placing in a room with constant temperature of 23° and constant humidity of 30 per cent. After 40 hours the preparations were ground and well mixed and allowed to equilibrate for 24 hours longer, at which time they had come to constant weight.

Samples for all analyses were taken in closed vessels in the room in which the preparations had been air-dried. Carbon and hydrogen were determined by the micromethod of Pregl (13). Nitrogen was determined by the micro-Dumas procedure, by macro-Kjeldahl according to the procedure here described, and by the procedure of Campbell and Hanna (9), modified by continuing the digestion for 2 hours, instead of 12 minutes, after the digest cleared.[6] Moisture content was determined by heating in an air oven at 102° to constant weight. Ash was determined on the residues from carbon determinations. Values for C, H, and N were corrected for moisture content of the air-dried preparations and for ash when present. Hydrogen was further corrected for the hydrogen of the water content. The results are shown in Table I.

The results obtained by the macro-Kjeldahl procedure with mercury as catalyst checked with those by the Dumas method within the limits of error of duplicate analyses. Macro-Kjeldahl analyses by the procedure of Campbell and Hanna (9) even when digestion was prolonged for 2 hours after clearing, gave only 97 per cent of the nitrogen determined by the Dumas method.

TABLE I

Elementary Analyses of Total Plasma Proteins Prepared from Dried Pooled Human Plasma and from Fresh Dog Plasma

Analysis	Procedure used for analysis	Source of total plasma protein preparation			Average for human plasma
		Fresh dog plasma	Dried pooled human plasma, I	Dried pooled human plasma, II	
		per cent of dry weight	*per cent of dry weight*	*per cent of dry weight*	
Ash		0.14	0	0.31	
Moisture		11.78	9.84	9.37	
Carbon*	Combustion	53.25	53.57	53.25	53.41
Hydrogen†*	"	6.97	7.05	7.15	7.10
Nitrogen*	Dumas	16.38	15.96	16.08	16.02
"	Macro-Kjeldahl with Hg	16.33	16.07	16.04	16.06
"	Campbell and Hanna method with Se (9)	15.90	15.42		
Factor $\frac{\text{protein}}{\text{N}}$ for macro-Kjeldahl with Hg catalyst		6.12	6.22	6.24	6.23

* Corrected for moisture and ash content.

† Corrected for hydrogen in H_2O content of preparations.

Recovery of Ammonia by Macro Distillation in Presence of Mercury

As controls for various procedures 10 ml. portions of 1 per cent solution of ammonium sulfate or 20 ml. of M/14 ammonium chloride solution were diluted with water and distilled, after addition of 5 ml. of 18 N NaOH, into 50 ml. of 4 per cent boric acid, and titrated with N/14 sulfuric acid. The amounts of ammonia titrated in the distillates were taken as "100 per cent," for comparison with the amounts of ammonia obtained when mercury and other reagents were added. Distillations of ammonium sulfate or ammonium chloride were then performed in the presence of K_2SO_4, H_2SO_4, and HgO in the amounts used in the macroprocedure described, with addition of potassium sulfide, sodium thiosulfate, or zinc dust to prevent retention of ammonia by the mercuric oxide. The results are shown in Table II.

---

[6] The digestion mixture of Campbell and Hanna is made by dissolving 10 gm. of $CuSO_4 \cdot 5H_2O$ and 10 gm. of selenium oxide in a mixture of 250 ml. of syrupy H_3PO_4 and 750 ml. of concentrated H_2SO_4. Of this, 20 ml. are used for each macroanalysis.

TABLE II

Recovery of Ammonia in Macro Distillation of Ammonium Salts in Presence of H_2SO_4, K_2SO_4, $HgSO_4$, and 18 NNaOH, with Various Procedures for Preventing Retention of Ammonia by HgO

Substance distilled	No. of determinations	Procedure for treating mercury	Ammonium distillate, mean yield
			per cent
$(NH_4)_2SO_4$	3	25 ml. 4% K_2S added to acid mixture *before* NaOH	98.2
"	3	2.5 gm. solid $Na_2S_2O_3 \cdot 5H_2O$ added *after* NaOH	98.0
"	3	2.5 " " " " *before* "	99.7
"	2	5% $Na_2S_2O_3 \cdot 5H_2O$ dissolved *with* NaOH	99.4
NH_4Cl	7	2 gm. zinc dust added *before* NaOH	100.0

Effect of Digestion Rate and Time on Macro-Kjeldahl Procedure

2 ml. samples of plasma were analyzed by the macro-Kjeldahl procedure described in this paper. When plasma was digested with rapid boiling, so that clearing occurred in 15 to 18 minutes, and boiling was continued for 0.5 and 1 hour after clearing, the results were 99.2 and 99.0 per cent of those by the procedure as described, in which "subboiling" was used throughout, and was continued for 2 hours after the digest became clear. With "subboiling" the preclearing period was 30 to 92 minutes. When the preclearing period required 50 to 92 minutes, maximum results were obtained even when the postclearing time was shortened to less than 2 hours. Digestion prolonged more than 2 hours after clearing gave no higher results than a 2 hour period. The blank determinations for the 8 hour digestion were twice as high as those for the 2 hour digestion. The results are shown in Table III.

TABLE III

Analyses of 2 Ml. Samples of Plasma for Total Nitrogen by Present Macro-Kjeldahl Procedure, Varying Digestion Rate and Time

Digestion before clearing		Digestion after clearing		Total digestion time	N found	Average N found	Per cent of digestion by subboiling with 30 min. to clear and 2 hrs. after clearing
Time	Conditions	Time	Conditions				
min.		*hrs.*		*hrs.*	*mg.*	*mg.*	
15–18	Boiling	0.5	Boiling	0.75	21.07		
					21.07		
					21.35	21.14	99.2
15–18	"	1	"	1.25	21.29		
					21.24		
					20.80	21.11	99.0
50	Subboiling	0.5	Subboiling	1.25	21.43		
					21.39		
					21.43		
					21.21	21.37	100.2
92	"	0.5	"	2	21.36		
					21.07		
					21.15		
					21.46	21.26	99.7
92	"	1	"	2.5	21.38		
					21.40		
					21.38	21.39	100.3
30	"	1	"	1.5	21.07		
					21.07		
					21.02		
					21.15	21.08	98.9
30	"	2	"	2.5	21.23		
					21.33		
					21.34		
					21.36	21.32	100.0
30	"	4	"	4.5	21.31		
					21.17		
					21.01		
					21.37	21.22	99.5
30	"	8	"	8.5	21.14		
					21.49		
					21.34		
					21.28	21.31	100.0

Effect of Selenium in Addition to Mercury As Catalyst in Macro-Kjeldahl Procedure

Dog plasma was analyzed by the macroprocedure as described, with mercury as catalyst, and by the same procedure with addition of 0.15 gm. of selenium powder.

It appears from Table IV that selenium and mercury together give digestion not significantly more rapid or complete than mercury alone.

Comparison of Methods for Determination of Total Nitrogen of Plasma by Various Macro-Kjeldahl Procedures

An approximately 7 per cent solution of Lyovac (lyophilized pooled human plasma, Sharp and Dohme), two pooled dog plasmas, and a pooled human plasma were digested by the method described in this paper with mercury as catalyst, and by the procedure of Campbell and Hanna (9) with Se and Cu as catalysts. In distillation of the digests containing mercury, thiosulfate was used instead of zinc dust, as these analyses were done before the use of zinc had been adopted. As shown in Table I, the ammonia yields from the digests with mercury might have been slightly higher if zinc had been used in the distillation. The results in Table V indicate that the digest mixture with mercury gave higher results than any of the other mixtures. The difference might have been still a little greater if zinc had been used in the distillation.

TABLE IV

Total Nitrogen of Plasma by Macro-Kjeldahl with Mercury As Catalyst with and without Addition of Selenium; Zinc Dust to Remove HgO during Distillation

Source of plasma used (2 ml. samples)	Time digested after clearing	Catalysts used					
		Mercuric oxide			Mercuric oxide + selenium		
		Time required for digest to clear	N per 2 ml. plasma	N, per cent of 2 hr. digestion	Time required for digest to clear	N per 2 ml. plasma	N, per cent of 2 hr. digestion *without* Se
	hrs.	*min.*	*mg.*		*min.*	*mg.*	
Dog	0	14*	17.58	98.9	12*	17.55	98.7
	0	27†	17.54	98.6	25†	17.52	98.6
	0.5	†	17.73	99.7	†	17.73	99.7
	2.0	†	17.78	100.0	†	17.72	99.7
Human	0.5	40†	19.23	98.7	40†	19.36	99.3
	2.0	40†	19.49	100.0	40†	19.41	99.6

* Boiling.

† Subboiling.

Recovery of Ammonia from NH_4Cl by Steam Distillation in Presence of All Reagents Used for Micro-Kjeldahl Procedure with Mercury As Catalyst

Samples of standard NH_4Cl solutions, containing respectively 1, 0.5, and 0.2 mg. of nitrogen, were measured into micro-Kjeldahl digestion tubes.

All reagents used for micro digestion were added and the contents of the tubes were distilled as described in the microprocedure. The ammonia was received into 10 ml. of 0.01428 N H_2SO_4. The volume of distillate collected was 50 ml.; this was the volume found adequate for quantitative distillation of ammonia in the still that was used. (The volume of distillate required was determined by distilling samples of NH_4Cl containing 4 mg. of nitrogen, collecting varying volumes of distillate, from 25 to 50 ml. 25 ml. of distillate showed 99.6 per cent of the theoretical value, and 50 ml. gave theoretical values.)

TABLE V

Comparison of Methods for Determination of Total Nitrogen of Plasma by Various Macro-Kjeldahl Procedures

Source of plasma used (2 ml. samples)	Time digested after clearing	Procedure used									
		Digest, 10 gm. K_2SO_4, 1 gm. HgO, 20 ml. H_2SO_4. Distillation with sodium thiosulfate dissolved in				Digestion mixture of Campbell and Hanna (9) with Se and Cu as catalysts		10 gm. K_2SO_4, 0.2 gm. $CuSO_4 \cdot 5H_2O$, 20 ml. H_2SO_4		10 gm. K_2SO_4, 0.2 gm. $CuSO_4 \cdot 5H_2O$, 20 ml. H_2SO_4, 0.5 ml. H_2O_2	
		Added NaOH		Diluted acid digest, then NaOH added							
		N in sample analyzed	Per cent of 2 hr. digestion with HgO	N in sample analyzed	Per cent of 2 hr. digestion with HgO	N in sample analyzed	Per cent of 2 hr. digestion with HgO	N in sample analyzed	Per cent of 2 hr. digestion with HgO	N in sample analyzed	Per cent of 2 hr. digestion with HgO
	hrs.	*mg.*		*mg.*		*mg.*		*mg.*		*mg.*	
Solution of Lyovac*	0.5			17.11	99.1	16.76	98.0				
	1			17.18	99.5						
	2			17.27	100.0	17.03	98.6				
	4					17.03	98.6				
Pooled dog Plasma 1	0.5					22.50	97.1				
	1					22.71	98.0				
	2	23.16	100.0			22.85	98.5				
Pooled dog Plasma 2	0.5	22.63	99.8								
	2	22.68	100.0			22.37	98.6				
	4					22.54	99.3				
	8					22.63	99.7				
Pooled human	2			23.03	100.0						
	4					22.65	98.4	22.13	96.1	22.06	95.8

* Lyophylized pooled human plasma, Sharp and Dohme.

The recovery of ammonia by distillation of samples of NH_4Cl containing 0.2 to 1 mg. of nitrogen varied from 99.6 to 100.4 per cent of the theoretical values, as shown in Table VI.

TABLE VI

Recovery of Ammonia from NH_4Cl by Steam Distillation in Presence of All Reagents Used for Present Titrimetric Micro-Kjeldahl Procedure with Mercury As Catalyst

Nitrogen in NH_4Cl sample	
Theoretical	Recovered in distillate
mg.	*per cent*
1	100.0
1	99.8
1	99.8
0.5	100.4
0.5	100.0
0.5	99.6
0.2	100.0
0.2	100.0

TABLE VII

Determination of Total Nitrogen of Plasma by Macro and by Micro Titrimetric Kjeldahl Procedures Described

Procedure used	Plasma in sample analyzed	N found in sample analyzed		N found per 100 ml. plasma		N found in per cent of result by macro-procedure	
		Replicates	Mean	Replicates	Mean	Replicates	Mean
	ml.	*mg.*	*mg.*	*mg.*	*mg.*		
Macro	2	19.38		969.0			
	2	19.35		967.5			
	2	19.34		967.0			
	2	19.37	19.36	968.5	968.0		100.0
Micro	0.2	1.932		966.0		99.8	
	0.2	1.932		966.0		99.8	
	0.2	1.940	1.935	970.0	967.3	100.2	99.9
“	0.1	0.972		972.0		100.4	
	0.1	0.968		968.0		100.0	
	0.1	0.978		978.0		101.0	
	0.1	0.970	0.972	970.0	972.0	100.2	100.4
“	0.05	0.484		968.0		100.0	
	0.05	0.482		964.0		99.6	
	0.05	0.482		964.0		99.6	
	0.05	0.488	0.484	976.0	968.0	100.8	100.0
“	0.02	0.198		990.0		102.3	
	0.02	0.198		990.0		102.3	
	0.02	0.196	0.197	980.0	986.7	101.2	101.9

Determination of Total Nitrogen of Plasma by Present Macro and Micro Titrimetric Kjeldahl Procedures

The plasma used for the analyses was an approximately 7 per cent solution of Lyovac (a lyophilized pooled human plasma prepared by Sharp and Dohme). For macro-Kjeldahl analyses samples of 2 ml. were taken. For micro-Kjeldahl analyses 10- and 50-fold dilutions of the plasma were prepared with 0.85 per cent sodium chloride solution. Samples as designated in Table VII were taken for analyses, covering a range of approximately 0.2 to 2 mg. of nitrogen. The results are given in Table VII.

TABLE VIII

Total Nitrogen Determined in Samples of 0.1 Ml. of Pooled Plasma by Present Gasometric Micro-Kjeldahl Procedure

P_{N_2}	Temperature	Factor	Nitrogen in sample	
			Found	Per cent of N by macromethod*
mm.	°*C.*		*mg.*	
377.8	23.0	0.003156	1.192	100.1
377.4	23.3	53	1.190	99.9
377.8	23.5	50	1.190	99.9
378.0	24.0	45	1.189	99.8
377.4	24.1	44	1.187	99.7
378.5	24.4	41	1.189	99.8
379.0	24.5	40	1.190	99.9
378.8	24.6	39	1.189	99.8
Mean........			1.190	99.9

* Nitrogen by macro-Kjeldahl was 11.91 mg. per ml.

Comparison of Total Nitrogen of Plasma Determined by Present Macro Titrimetric and Micro Gasometric Procedures

2 ml. samples of pooled human plasma were analyzed by the macro-Kjeldahl procedure. The mean result was taken as 100 per cent for comparison with results by the microprocedure. For the microanalyses the plasma was diluted 10-fold, and 1 ml. samples of the dilute solution were taken for analysis. The results are given in Table VIII.

DISCUSSION

Nitrogen Content of Plasma Proteins—Total plasma proteins prepared from two pools of human plasma, analyzed for total nitrogen by the macro-Kjeldahl procedure described, with mercury as catalyst, gave a mean value of 16.06 per cent, which checked with the mean value of 16.02 per cent by the Dumas method. The macro-Kjeldahl procedure of Campbell and Hanna (9) with digestion for 2 hours after clearing and with Se and Cu as catalysts gave a value of 15.42 per cent.

The literature on the analysis of plasma proteins reports varying nitrogen percentages. The lack of agreement is in part probably due to absence of exact data on the moisture and ash contents of the proteins analyzed. Proteins are so hygroscopic that completely dried samples cannot be weighed with accuracy. The most uniform analyses are obtained, according to the writers' experience, when the protein is air-dried by equilibration with the moisture of the atmosphere, and samples are weighed for all analyses, including moisture and ash, during a short time interval and under the atmospheric humidity and temperature conditions of equilibration. In part it appears that the inconstant and usually low nitrogen values in the literature may have been due to the use of inadequate digestion procedures for Kjeldahl analyses.

Bierry and Vivario (24) reported a mean nitrogen value of 15.26 per cent for preparations of total human plasma proteins. They did not give ash or moisture content, and did not indicate the procedure for the nitrogen analyses. Block and his associates (25–27) reported numerous analyses of total proteins of human serum, giving nitrogen values varying from 14.3 to 15.3 per cent. Analyses were performed by macro-Kjeldahl, but the procedure used was not stated. Some of the samples were dried at 110° (25, 27); other results (26) are given without mention of drying or of ash or moisture content. Murrill, Block, and Newburgh (28) prepared proteins from a pool of three human sera, but did not determine ash or moisture content. Nitrogen determined by the micro-Kjeldahl procedure of Pregl was 14.28 per cent. Robinson and Hogden (23) prepared proteins from three human sera. Dried ash-free samples were weighed. Nitrogen determined by macro-Kjeldahl, with a digestion mixture of H_2SO_4, H_3PO_4, and $SeOCl_2$, gave values between 15.5 and 16.0 per cent, mean 15.72. Cook (29) prepared proteins from four pools of human plasma. Dried samples were weighed. Nitrogen determined by the micro-Kjeldahl procedure of Chibnall, Rees, and Williams (30), with H_2SO_4, K_2SO_4, $CuSO_4$, and Na_2SeO_4, gave values between 14.71 and 15.54 per cent, mean 15.23. Nitrogen determined by these authors by micro-Dumas gave values between 13.29 and 15.96 per cent, mean 15.20. No mention is made of ash content. They concluded that the proper factor for conversion of nitrogen to total plasma protein was 6.6. Brand, Kassell, and Saidel (31) and Brand (32) analyzed fractions of human plasma proteins for nitrogen by the micro-Dumas method, in which they used air-dried samples and corrected for ash and moisture content. In the fractions which were pure (albumin and γ-globulin) they found 15.95 per cent nitrogen for albumin and 16.03 for γ-globulin. The factors for conversion of nitrogen to protein calculated from these values are 6.27 for albumin and 6.24 for γ-globulin, agreeing closely with the factor found in the present paper for total proteins of normal human plasma.

The factor 6.25, for conversion of nitrogen to protein, has been generally used for at least 75 years. Its origin is obscure and difficult to trace. It has long been known that different proteins vary considerably in their nitrogen content, and that for each protein its own factor must be established before exact calculation of the weight of dry protein present can be made from nitrogen determinations. From the data given in this paper for total human plasma proteins, and the data of Brand and his associates for pure human plasma albumin and γ-globulin, the factor 6.25 seems justifiable, for the present, for the calculation of weights of plasma proteins from nitrogen.

Some of the markedly higher values for the factor for plasma proteins, derived from other analyses in the literature, appear to be attributable to low nitrogen values by the Kjeldahl procedures used. For our own protein preparations the factor calculated from the determination of nitrogen by the procedure of Campbell and Hanna (9) is 6.49, because the procedure gave nitrogen values 3 per cent lower than the Dumas combustion or Kjeldahl digestion with mercury as catalyst.

Losses with Selenium and Mercury As Catalysts—The literature indicates that when selenium is used as a catalyst for the Kjeldahl procedure the length of time for digestion should be carefully established for the substance analyzed. Sandstedt (33) reported that the loss of nitrogen on long heating with selenium is greater than with other catalysts. Davis and Wise (34) and Illarionov and Ssolovjeva (35) reported a loss of nitrogen if heating was longer than 35 minutes with selenium. Dalrymple and King (36) found that digestion for 1 hour with HgO and K_2SO_4 gave the same result as digestion for 3 to 6 hours with selenium or 1.5 hours with various selenates. With selenates the recovery of nitrogen rose with time of digestion to a maximum, then diminished with progressive loss of nitrogen. Patel and Sreenivasan (37) reported progressive losses of nitrogen during digestion of casein with selenium or selenium plus mercuric oxide as catalysts if digestion was prolonged beyond 15 minutes after clearing. Osborn and Krasnitz (38) reported that when the digestion period was extended danger of loss of nitrogen with different catalysts increased in the order of Hg, Se, Se + Hg. Use of mercury involved the least danger of loss.

Determinations of carbon, hydrogen, and of nitrogen by the Dumas method were performed by Dr. A. Elek, to whom we wish to express our gratitude.

SUMMARY

Only digestion mixtures containing mercury as catalyst have been found to give nitrogen values for proteins as high as the values yielded by Dumas combustion.

Methods are described in which digestion mixtures yielding nitrogen values equal to those of the Dumas combustion are employed, and in which the ammonia formed is determined by macro or micro titration, or by micro gasometric measurement.

BIBLIOGRAPHY

1. Kjeldahl, J., *Z. anal. Chem.*, **22**, 366 (1883); *Compt.-rend. trav. Lab. Carlsberg*, **2**, 1 (1883); **2**, 197 (1888).
2. Vickery, H. B., *Yale J. Biol. and Med.*, **18**, 473 (1946).
3. Friedrich, A., *Mikrochemie*, **13**, 91 (1933).
4. Bradstreet, R. B., *Chem. Rev.*, **27**, 331 (1940).
5. Gunning, J. W., *Z. anal. Chem.*, **28**, 188 (1889).
6. Wilfarth, H., *Chem. Zentr.*, **16**, 17, 113 (1885).
7. Arnold, C., *Chem. Zentr.*, **17**, 337 (1886); *Arch. Pharm.*, **24**, 785 (1886).
8. Arnold, C., and Wedemeyer, K., *Z. anal. Chem.*, **31**, 525 (1892).
9. Campbell, W. R., and Hanna, M. I., *J. Biol. Chem.*, **119**, 1 (1937).
10. Böttcher, O., *Landw. Versuch.-Sta.*, **41**, 170 (1892).
11. Sörensen, S. P. L., and Andersen, A. C., *Z. physiol. Chem.*, **44**, 429 (1905).
12. Miller, L., and Houghton, J. A., *J. Biol. Chem.*, **159**, 373 (1945).
13. Pregl, F., and Roth, H., Die quantitative organische Mikroanalyse, Berlin, 4th edition, 105 (1935).
14. Meeker, E. W., and Wagner, E. C., *Ind. and Eng. Chem., Anal. Ed.*, **5**, 396 (1933).
15. Van Slyke, D. D., and Kugel, V. H., *J. Biol. Chem.*, **102**, 489 (1933).
16. Andersen, A. C., and Jensen, B. N., *Z. anal. Chem.*, **67**, 427 (1925–26).
17. Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, **136**, 509 (1940).
18. Van Slyke, D. D., Hiller, A., Weisiger, J. R., and Cruz, W. O., *J. Biol. Chem.*, **166**, 121 (1946).
19. Parnas, J. K., and Wagner, R., *Biochem. Z.*, **125**, 253 (1921).
20. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61**, 523 (1924).
21. Van Slyke, D. D., *J. Biol. Chem.*, **73**, 121 (1927).
22. Van Slyke, D. D., *J. Biol. Chem.*, **71**, 235 (1926–27).
23. Robinson, H. W., and Hogden, C. G., *J. Biol. Chem.*, **140**, 853 (1941).
24. Bierry, H., and Vivario, R., *Compt. rend. Soc. biol.*, **89**, 15 (1923).
25. Block, R. J., Darrow, D. C., and Cary, M. K., *J. Biol. Chem.*, **104**, 347 (1934).
26. Block, R. J., *J. Biol. Chem.*, **133**, 71 (1940).
27. Block, R. J., Jervis, G. A., Bolling, D., and Webb, M., *J. Biol. Chem.*, **134**, 567 (1940).
28. Murrill, W. A., Block, W. D., and Newburgh, L. H., *J. Biol. Chem.*, **133**, 521 (1940).
29. Cook, R. P., *Biochem. J.*, **40**, 41 (1946).
30. Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, **37**, 354 (1943).
31. Brand, E., Kassell, B., and Saidel, L. J., *J. Clin. Invest.*, **23**, 437 (1944).

32. Brand, E., *Ann. New York Acad. Sc.*, **47,** 187 (1946). Brand, E., and Edsall, J. T., in Annual review of biochemistry, Stanford University, **16,** 223 (1947).
33. Sandstedt, R. M., *Cereal Chem.*, **9,** 156 (1932).
34. Davis, C. F., and Wise, M., *Cereal Chem.*, **10,** 488 (1933).
35. Illarionov, V. V., and Ssolovjeva, N. A., *Z. anal. Chem.*, **100,** 328 (1935).
36. Dalrymple, R. S., and King, G. B., *Ind. and Eng. Chem., Anal. Ed.*, **17,** 403 (1945).
37. Patel, S. M., and Sreenivasan, A., *Anal. Chem.*, **20,** 63 (1948).
38. Osborn, R. A., and Krasnitz, A., *J. Assn. Official Agr. Chem.*, **17,** 339 (1934.)

DETERMINATION OF PROTEIN IN URINE BY THE BIURET METHOD

By ALMA HILLER, ROGER L. GREIF, and WILLIAM W. BECKMAN

With the Technical Assistance of John Plazin

(*From the Hospital of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, July 15, 1948)

A method for the determination of protein in urine by means of the biuret reaction was described by Hiller (1) and by Hiller, McIntosh, and Van Slyke (2), in which pure biuret prepared by Kahlbaum was used as a standard for visual colorimetry. Since Hiller (1) the biuret method has been used for the determination of urinary proteins by Price (3) and by Lehmann (4), and has been widely used in many forms and modifications for the determination of plasma proteins. A review of the literature on the biuret method will not be given here, but may be found in the papers of Küntzel and Dröscher (5) and of Robinson and Hogden (6). The latter workers studied the conditions necessary for the production of a stable color which bears a quantitative relationship to the protein concentration and also published transmission curves for the biuret color.

The present paper describes a photometric method for the determination of urinary protein in which the entire procedure is carried out in a single test-tube, which serves as a cuvette for the photoelectric spectrophotometer. The method can be used even when the urine contains Evans blue excreted after blood volume determinations. Since pure preparations of biuret are not now obtainable on the market, solutions of urinary proteins, of concentration determined by accurate Kjeldahl analysis, are used to prepare standard optical density curves, and the use of a chrome-alum solution for checking the curves is detailed.

METHOD

Apparatus

Cuvettes, 15 by 100 mm. (outer measurements). Test-tubes, thick wall, without lip, from the Arthur H. Thomas Company, No. 9446, make good inexpensive cuvettes. After cleaning with dichromate cleaning mixture the tubes are tested for uniformity by reading the optical density of a solution containing approximately 15 gm. of $CuSO_4 \cdot 5H_2O$ per 100 ml. in each tube in a spectrophotometer at a wave-length of 560 mμ. Tubes which read within an optical density variation of ±0.0015 of the average are chosen as cuvettes. These are then etched with numbers and calibrated to contain 10 ml. The tubes must be carefully handled to prevent scratching. Before each series of readings in the spectrophotometer, finger-marks must be removed by wiping the outside of the tube first with a damp, then with a dry towel. They must be cleaned immediately after use. A good cleaning procedure is to remove precipitates by rinsing and shaking with water; then small amounts of copper sulfate which adhere to the upper part of the inner walls of the tubes are removed with dilute hydrochloric acid, approximately 1 N. The tubes are then rinsed five times with tap water and three or four times with distilled water and inverted in a test-tube rack to drain onto a towel. Only the tubes used for blank determinations need be dry. The tubes used for protein analysis can be used without drying.

Rubber stoppers, solid No. 0, to fit cuvettes.

Centrifuge metal shields $3\frac{1}{8}$ inches long and $\frac{3}{4}$ inch in diameter will fit the size of cuvettes used. With an eight place head and three or four place trunnion carriers a large number of tubes can be centrifuged at one time.

Photoelectric spectrophotometer. The Coleman junior model was used, but any type can be used, with a cuvette holder 3 inches in length. If the holder is too wide for the cuvettes, a ring of hard rubber can be fitted into the top of the holder to keep the cuvettes in a vertical position.

A 5 ml. burette marked at 0.25 ml. intervals to deliver the copper sulfate solution.

A 25 ml. burette to deliver trichloroacetic acid.

Dispenser for sodium hydroxide. An aspirator bottle or a separatory funnel type of vessel, equipped with a soda lime tube and an outlet tube with a small opening for delivery of small drops.

Reagents

Trichloroacetic acid, 10 per cent solution. Keep in the refrigerator when not in use.

Copper sulfate, 20 gm. of $CuSO_4 \cdot 5H_2O$ per 100 ml.

Sodium hydroxide, reagent grade, 3 per cent solution. This solution is stored in the dispenser described under "Apparatus," and should not be allowed to age beyond 2 weeks, as the solution on standing over a longer period of time in glass gives higher blanks and lower protein values than when freshly made (see Table III).

Procedure

If the urine contains a precipitate, it should be filtered before analysis.

Precipitation of Protein—Enough urine to contain between 5 and 20 mg. of protein is pipetted into the bottom of each cuvette. The samples of albuminous urine usually range from 0.5 to 5 ml. to contain the desired amount of protein. If the concentration of protein is higher than 30 mg.

per liter, the urine is diluted with water so that a sample will contain between 5 and 20 mg. of protein. To the urine in each cuvette an equal volume of 10 per cent trichloroacetic acid is added. If the total volume of urine plus trichloroacetic acid is 6 ml. or less, mixing can be accomplished by shaking the cuvette. If the volume is greater than 6 ml., a thin stirring rod should be used to get complete mixture, with care not to scratch the wall of the cuvette. The rod is washed down with a few drops of water. The cuvette is allowed to stand 10 minutes, then centrifuged at about 2500 R.P.M. for 10 minutes. The supernatant solution is decanted and the cuvette is inverted against a towel or filter paper for a moment to drain.

Dissolving Protein Precipitate—The protein precipitate is dissolved by adding 2 ml. of 3 per cent sodium hydroxide. The cuvettes are shaken occasionally until solution is complete, but shaking must be gentle to avoid formation of foam. If cuvettes containing appreciable amounts of protein are allowed to stand for a time without occasional shaking, undissolved protein at the bottom of the cuvette will form a clear gel and may be overlooked, as it is difficult to see and requires a longer time to dissolve than does the white precipitate. If the cuvette is examined with a window or light as background, while whirling gently, a spiral of dissolving protein can be seen rising from the bottom of the cuvette. Solution is complete when there are no transparent strands on shaking the solution. More 3 per cent sodium hydroxide solution is then added with occasional shaking, until the volume in the cuvette reaches the 10 ml. mark.

Two dry cuvettes are filled to the 10 ml. mark with 3 per cent sodium hydroxide to serve as blanks.

Setting Zero Point of Photometer—The zero optical density point is set before each reading with the holder in place, but without a cuvette (air zero).

Blanks—The density of the 3 per cent NaOH in the cuvettes is read as D_{B_1}, Blank 1.

Reading of Optical Density, D_1, Due to Pigments in Urine Carried Down by Precipitate of Protein—The tubes containing redissolved protein are wiped and the optical density is read as D_1 at wave-length 560 mμ.

Development of Biuret Color and Reading of Optical Density, D_2—After D_1 readings have been made, 0.25 ml. of 20 per cent copper sulfate is added from the 5 ml. marked burette to each protein solution and blank. Each tube is stoppered as soon as the copper sulfate has been added, and is immediately shaken vigorously about 15 times. Unless shaking is prompt, clumps of copper hydroxide may stick to the sides. Stoppers are removed and washed. The tubes are allowed to stand 10 minutes for the biuret color to develop, and are then centrifuged for 4 minutes at about 2500 R.P.M. (Blanks should not stand longer than 10 minutes before centrifugation as they tend to be lowered on standing.) They are then wiped clean and the optical density is read at 560 mμ, with the zero point set as described above. The readings of the protein solutions are recorded as D_2 and the blanks are D_{B_2}, Blank 2. Tubes should be washed immediately after use as described under "Apparatus."

Calculation

D_1 = optical density reading of 10 ml. of protein solution
D_{B_1} = " " " " solvent (3 per cent NaOH) = Blank 1
D_2 = " " " " 10.25 ml. of protein solution + copper sulfate
D_{B_2} = optical density reading of reagent blank (NaOH + $CuSO_4$) = Blank 2
D_P = " " due to biuret color formed from protein in a volume of 10 ml.

$$D_P = 1.025\,(D_2 - D_{B_2}) - (D_1 - D_{B_1}) \tag{1}$$

The mg. of protein in the sample analyzed are read from a curve constructed for this purpose, relating D_P to mg. of protein for the specific set of cuvettes and spectrophotometer used.

$$\text{Mg. protein per liter urine} = \frac{\text{mg. protein in sample analyzed} \times 1000}{\text{ml. urine in sample taken}}$$

Construction of Curve Relating Optical Density to Mg. of Protein in Sample Analyzed—The relationship between optical density reading and protein concentration depends on the specific set of cuvettes and on the spectrophotometer used. It is therefore imperative that a curve be constructed for each set of apparatus.

Several urine samples containing protein can be used for this purpose. The urines are analyzed for total nitrogen and non-protein nitrogen by the macro-Kjeldahl procedure of Hiller, Plazin, and Van Slyke (7), who found that the best results of analyses of plasma protein were obtained when mercury was used as catalyst. For determination of non-protein nitrogen the protein is precipitated under the same conditions as for the biuret method; equal volumes of urine and 10 per cent trichloroacetic acid are mixed, let stand 10 minutes or longer, then centrifuged. Aliquot portions of the supernatant solution are taken for analysis.

The calculation for the protein content of the urines is

$$\text{Protein per 100 ml.} = 6.25\,[(\text{total N per 100 ml.}) - (\text{non-protein N per 100 ml.})]$$

Of each urine analyzed three or four samples of various size are chosen, containing amounts of protein ranging from about 3 to 15 mg. These are analyzed in triplicate by the biuret method, and the D_P values calculated by Equation 1 are plotted against the mg. of protein in the samples taken for analysis. A straight line curve can thus be constructed for calculations.

Such a curve was checked at frequent intervals and was found to remain constant over a period of 6 months.

EXPERIMENTAL

Choice of Wave-Length for Reading Biuret Color—Optical densities of the biuret color prepared from a sample of urine protein were read at various wave-lengths in the Coleman junior photoelectric spectrophotometer. A curve constructed from these data showed a maximum optical density at wave-lengths between 550 and 570 mμ, and was almost identical with the curve of Robinson and Hogden (6). A wave-length of 560 mμ was chosen.

Reproducibility of Results by Biuret Method—Urine samples were chosen which contained varying amounts of protein, between 3 and 19 mg. Twenty analyses were performed on each sample chosen. The results are shown in Table I.

TABLE I

Reproducibility of Results by Biuret Method for Various Amounts of Protein in Sample of Urine Analyzed

Urine sample	Protein found in sample; mean of 20 determinations	Standard deviation from mean	
ml.	*mg.*	*mg.*	*per cent of mean*
0.5	3.105	±0.065	±2.09
1	6.328	±0.077	±1.22
2	12.798	±0.126	±0.99
3	19.030	±0.171	±0.90

Stability of Biuret Color—On six urine specimens which were analyzed by the biuret method, optical density readings were taken within ½ hour after development of the biuret color. The cuvettes were stoppered and let stand at room temperature, 22°, in daylight but not in direct sunlight, and readings were repeated at 3 and 4 hours after color development. The results, recorded in Table II, show no appreciable change in optical density over a period of 4 hours.

Effect of Age of 3 Per Cent Sodium Hydroxide Solution on Results Obtained by Biuret Method—3 per cent sodium hydroxide solutions were protected from atmospheric CO_2 and were used after standing in glass containers for intervals up to 90 days, as indicated in Table III. For the analyses a solution of serum albumin containing 3.9 mg. of protein per ml. was used. Samples of 1 ml. were analyzed in triplicate for each sodium hydroxide solution. All the analyses in Table III were done on the same day. Reagent blanks were determined for each solution used. The sodium hydroxide solutions which were kept for 42 to 90 days all gave higher reagent blanks. The results shown in Table III indicate that the 3 per cent sodium hydroxide solution may be used for a period of 2 weeks without appreciably affecting the accuracy of the procedure.

TABLE II

Stability of Biuret Color on Standing at Room Temperature in Daylight but Not in Sunlight; Optical Densities with Air As Zero

Urine No.	Optical density readings; time after biuret color development		
	Within ½ hr.	After 3 hrs.	After 4 hrs.
Water	0.050	0.050	0.050
Reagent blanks	0.081	0.080	0.080
	0.081	0.080	0.080
152	0.441	0.440	0.440
153	0.391	0.391	0.391
154	0.233	0.233	0.234
155	0.349	0.349	0.350
156	0.300	0.300	0.300
164	0.590	0.590	0.590

TABLE III

Effect of Age of 3 Per Cent Sodium Hydroxide Solution on Biuret Results

Age of NaOH	Protein found in sample	Deviation from protein found with freshly made NaOH
days	*mg.*	*mg.*
0	3.9	
1	3.8	−0.1
2	3.9	0
3	3.9	0
4	3.8	−0.1
5	4.0	+0.1
6	3.7	−0.2
7	4.0	+0.1
13	3.8	−0.1
17	3.7	−0.2
19	3.7	−0.2
42	3.6	−0.3
49	3.7	−0.2
60	3.6	−0.3
90	3.7	−0.2

Correction for Urinary Pigments in Biuret Method—When protein is precipitated from a highly concentrated urine, some of the pigment is carried down with the precipitate. When such a precipitate is dissolved in alkali, the solution is sufficiently colored to give a slight optical density reading at the wave-length at which the biuret color is read.

To determine the amount of error introduced into the biuret method by this color, a serum albumin solution containing 10.6 mg. of protein per ml. was analyzed before and after addition of a dilute normal, straw-colored urine and of a concentrated normal, deeply colored urine. The results were calculated with and without the correction for the color of the protein solution (optical density readings D_1 in the method). Column 4 of Table IV shows that with varying amounts of protein in the sample the corrected readings indicate, within the limits of error of the method, the amount of protein present. When the calculations are made without correcting for pigment (Columns 5 and 6, Table IV), the results are increased to a degree beyond the experimental error of the method. This error becomes relatively greater when smaller amounts of protein are analyzed.

TABLE IV

Effect of Urinary Pigments on Biuret Method

Normal urine		Serum albumin solution	Protein in sample calculated from added albumin	Protein in sample by biuret method		Protein uncorrected, in per cent of corrected
Color	Sample taken (1)	(2)	(3)	Corrected for pigment (4)	Uncorrected for pigment (5)	(6)
	ml.	*ml.*	*mg.*	*mg.*	*mg.*	
	0	1	10.6	10.6	10.6	100.0
Dark	3	0.25	2.65	2.7	3.0	110.1
"	3	0.5	5.3	5.5	5.8	105.5
"	3	1	10.6	10.6	10.9	103.8
"	3	1.5	15.9	15.8	16.3	103.1
"	4	1	10.6	10.7	11.2	103.6
Light	4	1	10.6	10.6	10.9	102.8

The dye, Evans blue (T-1824), when injected into patients with proteinuria for the determination of plasma volume, is excreted bound to the urinary protein. A number of urines containing T-1824 were analyzed for protein by macro-Kjeldahl determination and by the biuret method. Results by the biuret method were calculated with and without the correction for dye (included in the D_1 readings). Table V shows that results calculated with the correction check with the results by Kjeldahl analysis within the limits of error, whereas the uncorrected photometric results are higher.

Comparison of Determinations of Urine Protein by Macro-Kjeldahl, by Biuret Method, and by Sedimentation Method of Shevky and Stafford (8) As Modified by MacKay (9)—Twelve urines were analyzed for protein (1) by the macro-Kjeldahl procedure described for construction of the curve for conversion of optical density to protein content, (2) by the biuret method, and (3) by the sedimentation method of Shevky and Stafford (8) as modified by MacKay (9). In Table VI the results by the biuret method are seen to check with those by macro-Kjeldahl analysis within the limits of error. The results by the sedimentation method show deviations from the Kjeldahl, ranging from −3.5 to +51.8 per cent.

TABLE V

Analyses of Urine Containing Evans Blue (T-1824) by Biuret Method

Urine specimen	Protein per liter			Biuret, per cent of Kjeldahl	
	Macro-Kjeldahl	Biuret method			
		Corrected for dye	Uncorrected for dye	Corrected for dye	Uncorrected for dye
	gm.	gm.	gm.		
B-251	13.8	14.0	14.5	101.5	105.0
B-147	33.0	33.0	33.5	100.0	101.5
Se	10.5	10.4	10.8	99.1	102.9
C	17.5	18.0	18.2	102.8	104.0
M-173	28.2	28.0	28.6	99.1	101.5
B-171	41.5	41.0	42.0	98.8	101.2
M-177	20.7	21.0	21.4	101.5	103.4

TABLE VI

Comparison of Determinations of Urine Proteins by Macro-Kjeldahl, by Biuret Method, and by Method of Shevky and Stafford

Urine specimen	Protein per liter			Per cent deviation from Kjeldahl	
	Macro-Kjeldahl	Biuret method	Shevky-Stafford	Biuret method	Shevky-Stafford
	gm.	gm.	gm.		
B-147	33.0	33.0	47.2	0	+43.0
B-251	13.8	14.0	18.7	+1.5	+35.5
B-171	41.5	41.0	63.0	−1.2	+51.8
M-173	28.2	28.0	34.5	−0.9	+22.4
N-177	20.7	21.0	28.8	+1.5	+39.0
R	8.0	8.0	10.4	0	+30.0
W	6.3	6.3	7.6	0	+20.5
O'F	25.6	25.2	24.7	−1.5	−3.5
Sp	3.6	3.6	3.8	0	+5.5
Ro	4.5	4.5	5.1	0	+13.3
C	17.5	18.0	18.2	+2.8	+4.0
Se	10.5	10.4	10.8	−0.9	+2.9

Procedures for Frequent Checking of Calculation Curve—To find a procedure for checking the calculation curve at frequent intervals a search was made for compounds which either give a biuret reaction or which give an optical density curve similar to that of the biuret color.

Chromic ammonium sulfate, $Cr_2(SO_4)_3(NH_4)_2SO_4 \cdot 24H_2O$, was chosen because in aqueous solution its transmission curve is near that of the biuret color, with a minimum transmission between wave-lengths of 570 and 590 mμ. Aqueous solutions of 0.5 and 0.25 per cent were prepared. These solutions showed a color instability when first made up. A daily increase was found in the optical density readings until the 4th day, but thereafter the readings remained unchanged when made at intervals up to 1 year. The optical density readings of the two solutions were located on the calculation curve and checked at intervals. Two preparations of chromic ammonium sulfate, one Baker's "analyzed," one a C.P. product obtained from the Fisher Scientific Company, gave the same results.

SUMMARY

A biuret method for the determination of urinary protein is described in which the entire procedure is carried out in a single test-tube which serves as a cuvette for the photoelectric spectrophotometer. Results agree with those by an accurate macro-Kjeldahl method (digestion with mercury catalyst), the standard deviation from the Kjeldahl values being of the order of ±1 per cent when the urine samples contained 6 to 19 mg. of protein.

BIBLIOGRAPHY

1. Hiller, A., *Proc. Soc. Exp. Biol. and Med.*, **24**, 385 (1926).
2. Hiller, A. McIntosh, J. F., and Van Slyke, D. D., *J. Clin. Invest.*, **4**, 235 (1927).
3. Price, J. W., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **140**, p. civ (1941).
4. Lehmann, J., *Nord. Med.*, **25**, 604 (1945).
5. Küntzel, A., and Dröscher, T., *Biochem. Z.*, **306**, 177 (1940).
6. Robinson, H. W., and Hogden, C. G., *J. Biol. Chem.*, **135**, 707 (1940).
7. Hiller, A., Plazin, J., and Van Slyke, D. D., *J. Biol. Chem.*, **176**, 1401 (1948).
8. Shevky, M. C., and Stafford, D. D., *Arch. Int. Med.*, **32**, 222 (1923).
9. MacKay, E. M., in Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry; Methods, Baltimore, 682 (1932).

SUBSTITUTES FOR SAPONIN IN THE DETERMINATION OF OXYGEN AND CARBON MONOXIDE OF BLOOD

By ALMA HILLER, JOHN PLAZIN, and DONALD D. VAN SLYKE

(*From the Hospital of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, July 15, 1948)

When oxygen or carbon monoxide in blood is determined by setting these gases free from HbO_2 or HbCO by means of ferricyanide, it is necessary to lake the cells completely, because ferricyanide does not penetrate the intact erythrocytes. The hemolytic agent used by Van Slyke and Neill (1) to lake the cells was saponin.

During the past few years commercial preparations of saponin which have been used as a hemolytic agent in the determination of oxygen in blood by the method of Van Slyke and Neill (1) have been found in this laboratory to give results considerably lower than those obtained with a Kahlbaum preparation of saponin which had been previously in use.

The use of Duponol WA and Duponol W-20 as a substitute for saponin in the determination of oxygen was reported by Vestling and Swerdlow (2). They used the method of Sendroy (3) for the determination of oxygen capacity, in which 32 per cent potassium ferricyanide containing the hemolytic agent is added to the blood after saturation of the blood with air (oxygen) in the chamber of the blood gas apparatus of Van Slyke and Neill (1). Ramsay (4) used oleyl sodium sulfate as a substitute for saponin, and warned that an excess causes stop-cocks to leak and impairs greatly the hemolytic action.

The presence and distribution of saponins in plants and their value as hemolytic agents have been investigated by Luft (5).

The present paper describes procedures in which urea or infusions of senega root, root soapwort, and quillaja soap bark, which are commercially available,[1] can be used as hemolytic agents in the determination of oxygen in blood.

Evidence is given that hemoglobin can be determined by the carbon monoxide capacity method of Van Slyke, Hiller, Weisiger, and Cruz (6) without addition of a hemolytic agent such as saponin.

METHODS

Determination of Oxygen in Blood with Urea As Hemolytic Agent

The method of Van Slyke and Neill (1) is used with slight modifications.

---

[1] Senega root, N. F. (cut and sifted), root soapwort (cut), and quillaja soap bark (cut) were obtained from the Amend Drug and Chemical Company, Inc., 117–119 East 24th Street, New York 10.

Reagents

Urea, 40 per cent solution, containing 1 per cent egg albumin.

Potassium ferricyanide, 32 per cent solution.

Sodium hydroxide, 1 N air-free solution.

Sodium hyposulfite, $Na_2S_2O_4$, air-free solution, described by Van Slyke (7).

Caprylic alcohol.

Procedure for 1 Ml. Samples of Blood

2 drops of caprylic alcohol are drawn into the capillary of the blood gas apparatus (1, 7), and 7.5 ml. of the urea solution are measured into the chamber. After making a mercury seal, the solution is deaerated by evacuating and shaking for 3 minutes. 6 ml. of the solution are run up into the cup; then 1 ml. of blood is measured into the chamber, followed by 1 ml. of the solution in the cup. After making a mercury seal, the mixture is evacuated and shaken 3 minutes to lake the blood. The vacuum is released, the upper cock being kept closed. In the cup of the gas apparatus place a few ml. of water and a little mercury. From a small rubber-tipped burette containing the 32 per cent potassium ferricyanide discard a drop so that there is no air space in the tip, insert the tip through the water and mercury into the bottom of the cup, and admit 0.2 ml. of the ferricyanide directly into the chamber. Seal with mercury, evacuate, and shake 3 minutes.

Carbon dioxide and oxygen are absorbed as usual (1), and pressure readings are taken with the meniscus at the 0.5 ml. mark.

Blanks are carried out by the procedure for blood, except that after deaeration of the urea solution 4 ml. are run up into the cup in place of 6 ml., and the blood sample is omitted. The ferricyanide is thus added to 3.5 ml. of deaerated urea solution in the chamber, instead of to a mixture of 2.5 ml. of that solution with 1 ml. of blood.

Determination of Oxygen in Blood with Senega Root, Root Soapwort, or Quillaja Soap Bark As Hemolytic Agents

Reagents

Sodium hydroxide, *sodium hyposulfite*, and *caprylic alcohol* are the same as described above.

1.6 per cent potassium ferricyanide in 0.5 per cent infusion of either senega root, root soapwort, or quillaja soap bark. The infusions are made by grinding the root or bark in a mortar or fine grinder and measuring 0.5 gm. into 100 ml. of water. The water is heated to boiling and the infusion is strained through finely woven cheese-cloth in a funnel.

Procedure

The procedure is that described by Van Slyke and Neill (1), except that the ferricyanide-saponin solution of these authors is replaced by a solution of ferricyanide in one of the above infusions.

EXPERIMENTAL

Determination of Oxygen in Blood—As controls by which to evaluate various procedures for the determination of oxygen in blood, blood was analyzed for oxygen capacity by the method of Van Slyke and Neill (1), except that the saponin-ferricyanide reagent contained 1.6 per cent potassium ferricyanide in place of 0.3 per cent. The saponin used was a Kahlbaum preparation which had long been in use in the laboratory and was known to give good results. The results by this procedure are recorded as 100 per cent in Table I.

A preparation of Baker saponin gave results which were 53.9 per cent of those with the Kahlbaum saponin when both were used in 0.3 per cent concentration in the ferricyanide solution. A preparation of saponin from the Amend Drug and Chemical Company, used in the same concentration, yielded 83.6 per cent. A concentration of 1 per cent yielded 98.8 per cent.

When water was substituted for the urea solution in the procedure described under "Methods," in which urea was used as hemolytic agent and the ferricyanide was added *after* laking of the blood, the yield was 97.3 per cent.

When 40 per cent urea was substituted for saponin, and the procedure was the same as for the controls above, the yield was 81.3 per cent and the blanks were high. When 1 per cent egg albumin was added in addition to urea, the yield was 92.8 per cent.

By the use of urea and egg albumin as described under "Methods," in which the blood is laked before addition of potassium ferricyanide, the yield was 99.5 per cent. The same procedure with omission of urea yielded 94.2 per cent.

Substitution of 0.5 per cent infusions of senega root, root soapwort, or quillaja soap bark for saponin gave results comparable to those with the Kahlbaum saponin. The results are shown in Table I.

Simultaneous Determination of Carbon Dioxide and Oxygen in Blood—The method of Van Slyke and Neill (1) was used, with the acid saponin-ferricyanide reagent described by Peters and Van Slyke (8). Two solutions are made: (*a*) 0.8 gm. of Kahlbaum saponin and 3.2 gm. of potassium ferricyanide diluted with water to 100 ml.; (*b*) 1 ml. of concentrated lactic acid of specific gravity 1.20 diluted to 100 ml. Before use equal volumes of the two solutions are mixed. Results with this reagent were used as controls by which to evaluate substitutes for saponin.

TABLE I

Effects of Replacing Kahlbaum Saponin by Other Hemolytic Agents in Determination of Oxygen Capacity of Blood

Blood No.	Hemolytic agent used	Procedure and reagents	Oxygen capacity		Mean in per cent of results with Kahlbaum saponin
			Duplicates	Mean	
			vol. per cent	*vol. per cent*	
1	Kahlbaum saponin	Van Slyke-Neill procedure with 0.3% saponin in 1.6% potassium ferricyanide as reagent	20.58 20.60	20.59	100.0
1	Baker saponin	" "	11.04 11.14	11.09	53.9
1	Amend saponin	" "	17.15 17.25	17.20	83.6
1	" "	Van Slyke-Neill with 1% saponin in 1.6% ferricyanide	20.26 20.34	20.30	98.8
1	Water	Water deaerated, blood added, shaken 3 min. to hemolyze, then 0.2 ml. 32% ferricyanide added	20.06 19.98	20.02	97.3
1	Urea	Van Slyke-Neill with 40% urea in 1.6% ferricyanide as reagent	16.72 16.75	16.74	81.3
1	Urea + egg albumin	Van Slyke-Neill with reagent containing 40% urea, 1% egg albumin, 1.6% ferricyanide	19.12 19.05	19.09	92.8
2	Kahlbaum saponin	Van Slyke-Neill with 0.3% saponin in 1.6% ferricyanide as reagent	18.98 18.98	18.98	100.0
2	Urea + egg albumin	40% urea in 1% egg albumin, deaerated, blood added, shaken 3 min. to hemolyze, then 0.2 ml. 32% ferricyanide added	18.86 18.92	18.89	99.5
2	Egg albumin	As above, urea omitted	17.92 17.85	17.89	94.2
2	Senega root	Van Slyke-Neill with 1.6% ferricyanide in 10% infusion of senega root as reagent	18.94 18.97	18.96	99.9
3	Kahlbaum saponin	Van Slyke-Neill with 0.3% saponin in 1.6% ferricyanide	22.89 22.90	22.90	100.0
3	Senega root	Van Slyke-Neill with 1.6% ferricyanide in 0.5% infusion of senega root as reagent	22.89 22.98	22.94	100.2
3	Root soapwort	Van Slyke-Neill with 1.6% ferricyanide in 0.5% infusion of root soapwort as reagent	22.82 22.80	22.81	99.6
3	Quillaja soap bark	Van Slyke-Neill with 1.6% ferricyanide in 0.5% infusion of quillaja soap bark as reagent	22.97 22.83	22.90	100.0

As substitutes for saponin, senega root and root soapwort were used. For these substitutes solution (*a*) was prepared by dissolving 3.2 gm. of potassium ferricyanide in 100 ml. of 2 per cent infusion of the root. The infusions were prepared as described under "Methods."

TABLE II

Effect of Using Substitutes for Saponin in Simultaneous Determination of Carbon Dioxide and Oxygen in Blood

Hemolytic agent used	CO_2 per liter blood		O_2 per liter blood		Per cent of result with Kahlbaum saponin	
	Replicates	Mean	Replicates	Mean	Mean CO_2	Mean O_2
	mM	*mM*	*mM*	*mM*		
Kahlbaum saponin	16.82		10.29			
	16.69		10.31			
	16.75	16.75	10.25	10.28	100.0	100.0
Senega root	16.77		10.33			
	16.78	16.78	10.32	10.33	100.1	100.5
Root soapwort	16.84		10.38			
	16.94	16.89	10.28	10.33	100.8	100.5

TABLE III

Determination of Carbon Monoxide Capacity of Blood by Method of Van Slyke, Hiller, Weisiger, and Cruz (6), with and without Saponin

Blood No.	Determination*	Hemolytic agent added to borax solution	Carbon monoxide capacity		Mean in per cent of results with Kahlbaum saponin
			Duplicates	Mean	
			vol. per cent	*vol. per cent*	
4	Total Hb	Kahlbaum saponin	22.04		
			21.94	21.99	100.0
4	" "	None	21.93		
			21.98	21.96	99.9
4	" "	None, but blood shaken 2 min. with borax solution before saturating with CO	22.00		
			21.98	21.99	100.0
5	Total Hb	Kahlbaum saponin	18.52		100.0
	" "	None	18.50		99.9
5	Active "	Kahlbaum saponin	18.24		100.0
	" "	None	18.24		100.0

* "Total Hb" is determined from the CO capacity of blood treated with $Na_2S_2O_4$; "active Hb" from CO capacity without $Na_2S_2O_4$ (6).

1 ml. samples of blood were analyzed and readings of carbon dioxide were taken with the meniscus at the 2 ml. mark, and of oxygen at the 0.5 ml. mark.

The results when senega root or root soapwort was used as a substitute for saponin differed by less than 1 per cent from the results obtained with the Kahlbaum saponin, as shown in Table II.

Determination of Carbon Monoxide Capacity of Blood Without Saponin—Hemoglobin was determined in blood by the carbon monoxide capacity method of Van Slyke, Hiller, Weisiger, and Cruz (6), with and without addition of Kahlbaum saponin to the borax solution. The results in Table III show that a hemolytic agent is not required for the procedure.

DISCUSSION

When urea as hemolytic agent in the determination of oxygen in blood is added simultaneously with the ferricyanide, as is saponin in the method of Van Slyke and Neill (1), low results are obtained, as shown in Table I. If, however, the blood is first laked in the urea solution before adding potassium ferricyanide, good results can be obtained. Blanks performed by this procedure, however, are high (10 mm. of P_{O_2} at the 0.5 ml. mark), and if such blanks are used the results are lowered. It was found, however, that when a protein, such as egg albumin, was added to the urea solution before addition of potassium ferricyanide, the blanks were lowered to 3 mm. of P_{O_2} at the 0.5 ml. mark. When egg albumin in concentration of 1 per cent was added to 40 per cent urea solution, and both blood and blank analyses were performed by the procedure as here described, the results obtained were comparable to those by the original procedure with a good saponin.

SUMMARY

Some commercial preparations of saponin currently obtainable, when used as hemolytic agents in the determination of oxygen in blood by the method of Van Slyke and Neill, give low results.

Infusions of senega root, root soapwort, and quillaja soap bark can be used as substitutes for saponin. Urea can be used in place of saponin if egg albumin is added to the urea solution and if the blood is laked in the urea-albumin solution *before* potassium ferricyanide is added.

In the determination of hemoglobin by the carbon monoxide capacity method of Van Slyke, Hiller, Weisiger, and Cruz, saponin can be omitted from the reagents without affecting the results. The successive actions of borax and acetate buffer appear to lake the cells sufficiently to make the HbCO completely accessible to the ferricyanide.

BIBLIOGRAPHY

1. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61**, 523 (1924).
2. Vestling, C. S., and Swerdlow, M. A., *Ind. and Eng. Chem., Anal. Ed.*, **16**, 581 (1944).

3. Sendroy, J., Jr., *J. Biol. Chem.*, **91**, 307 (1931).
4. Ramsay, W. N. M., *Biochem. J.*, **40**, 286 (1946).
5. Luft, G., *Monatsh. Chem.*, **47**, 259 (1926).
6. Van Slyke, D. D., Hiller, A., Weisiger, J. R., and Cruz, W. O., *J. Biol. Chem.*, **166**, 121 (1946).
7. Van Slyke, D. D., *J. Biol. Chem.*, **73**, 121 (1927).
8. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry; Methods, Baltimore (1932).

INTERACTIONS OF QUATERNARY AMMONIUM COMPOUNDS AND PROTEINS

A SIMPLE METHOD FOR THE RAPID ESTIMATION OF URINARY PROTEIN CONCENTRATIONS WITH ALKYLDIMETHYLBENZYLAMMONIUM COMPOUNDS*

By FRANCIS P. CHINARD

With the Technical Assistance of Dora M. Newell

(*From the Hospital of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, July 15, 1948)

A simple method of titration with a cationic detergent has been developed which permits rapid estimation of the concentration of urinary protein with a minimum of manipulations and time. The method depends on the formation of an insoluble anion-cation complex between quaternary ammonium ions, hereinafter referred to as the cationic detergent, and proteins, in the present instance mainly human albumin, at alkaline pH. No protein is precipitated from solution by the addition of the cationic detergent until a slight excess of this is present, at which time the solution shows faint but definite turbidity. Addition of more cationic detergent results at first in increasing turbidity, but as the addition is continued the turbidity decreases, almost as abruptly as it appeared, to give a nearly clear solution. Further addition of the cationic detergent is not attended by reappearance of turbidity. The end-point used in the present method is the appearance of a definite turbidity. The protein-quaternary ammonium ion complex formation is sensitive to ionic strength, non-electrolyte concentration, pH, and type of protein present (see, for example, Polonovski and Macheboeuf (1), Schmidt (2), and Valko (3)). For this reason, the method is restricted for accurate results to urine protein of the order of 2 or more gm. per liter (0.4 or more mg. of protein per sample) in order that the dilutions made may be sufficient to reduce the factors of urine ionic strength, non-electrolytes, and pH to negligible proportions. The method is, however, applicable to most cases of significant proteinuria. The simplicity of the procedure is indicated by the fact that we have been able to complete nearly 200 analyses in an afternoon.

If the end-point is determined by the optical density change in a photometer, the method can be used for samples containing as little as 0.02 mg. of protein.

---

* The preliminary phases of this work were started while the author was a Fellow in the Medical Sciences of the National Research Council in 1945-46.

Reagents

Alkyldimethylbenzylammonium chloride,[1] 0.1 per cent solution in distilled water. The solutions have been found to be stable for at least 3 months. This solution will be referred to as "the detergent."

2 N NaOH.

Procedure

Preparation of Standard Curve Relating Amount of Added Detergent to Amount of Protein in Sample—Because of the uncertain composition of the detergents available it is essential that a standard curve be prepared relating the amount of added detergent to the amount of protein in the sample. For the present work a series of twelve urine samples was selected for use as a standard. The protein concentration of the urines was calculated from the difference between total nitrogen and nitrogen after precipitation of the proteins with an equal volume of 10 per cent trichloroacetic acid. The technique used for digestion and distillation was that described by Hiller, Plazin, and Van Slyke (4). Turbidity titrations were then carried out as described below; the results were plotted with the protein concentration as ordinates and the ml. of detergent used as abscissae. An example of a standard curve with human serum albumin is given in Fig. 1.

Titration of Urine Proteins—0.1 or 0.2 ml. of the urine samples containing 0.4 or more mg. of protein is accurately pipetted into 2.5 × 10 cm. test-tubes, and 4 ml. of distilled water are added, followed by 0.2 ml. of 2 N NaOH. (The pH of the solution is approximately 13.) The detergent is then added from a 2 ml. burette, with swirling, until the appearance of a definite and permanent turbidity. This turbidity is most easily detected by titrating against a black background and by having a beam of light passing through the test-tube at right angles. Reproducibility may be insured by comparing with samples of the standards titrated previously, but after a few preliminary trials it will be found that the reproducibility of this turbidity end-point is no more diffcult than in ordinary acid-base titrations with phenolphthalein as an indicator. The mg. of protein present in the sample analyzed are then calculated from a standard curve, established from urines containing varying amounts of protein calculated from Kjeldahl N determinations.

---

[1] Unless otherwise specified, the results reported here have been obtained with myristamidopropyldimethylbenzylammomium chloride (trade name, Aerosol M), manufactured by the American Cyanamid Company, 30 Rockefeller Plaza, New York 20. For use 1 ml. of the concentrated detergent is diluted to 500 ml. in distilled water. We are indebted to the American Cyanamid Company for several samples of their product. Equally satisfactory and similar results have been obtained with cetyldimethylbenzylammonium chloride and dodecyldimethylbenzylammonium chloride in 0.1 per cent solutions. These last two compounds are available from the Onyx Oil and Chemical Company, Jersey City 2, New Jersey. A mixture of alkyldimethylbenzylammonium chlorides (alkyl = C_8H_{17} to $C_{18}H_{37}$), manufactured by the Winthrop Chemical Company, Inc., 170 Varick Street, New York 13, under the trade name Zephiran, may also be used in 0.1 per cent concentration, though it is slightly less satisfactory.

Calculations—A standard curve relating ml. of detergent to protein concentration is prepared as described above. The results for unknowns are read off the curve and the proper correction made for dilution of the urine.

Results

Standard Curve with Human Serum Albumin; Reproducibility of Results—Fig. 1 shows the relationship of the ml. of detergent required to reach the end-point and the mg. of human serum albumin present as determined by the Kjeldahl method. An average of six titrations was carried out for each point of the curve. The ml. of detergent added and the estimated standard deviations for each point were 0.519 ± 0.023, 0.863 ± 0.012, 1.220 ± 0.019, 1.528 ± 0.011, 1.834 ± 0.020, 2.224 ± 0.022. The dotted line in Fig. 1 (the extension of the straight line through the points) shows that the true end-point was overstepped by an absolute number of ml. for each point on the curve. The overstepping is equal to the X intercept of the extended straight line. This overstepping, equivalent to 0.18 ml. of detergent solution, is due to the fact that the first appearance of turbidity was not taken as the end-point, but rather the appearance of a definite turbidity, the reproducibility of which is indicated by the standard deviations above.

Comparison of Results Obtained by Turbidity Titration with Several Detergents by Biuret Reaction—In Fig. 2 are plotted the protein concentrations of urine of patients with the nephrotic syndrome, some of whom were receiving large amounts of human serum albumin intravenously. The results obtained by turbidity titration with myristamidopropyldimethylbenzylammonium chloride are plotted as ordinates, while the results obtained by the biuret reaction (5) and the Kjeldahl method (as described above under "Preparation of standard curve") are plotted as abscissae. Twelve Kjeldahl determinations of urine protein were used to calculate the equivalence of ml. of detergent and mg. of urinary protein. The estimated standard deviation of results by the turbidity titration from these by the Kjeldahl method was ±3.71 per cent. When the turbidity titration results were calculated as per cent of the biuret results, the mean for the 105 estimations was 100.5 per cent, with an estimated standard deviation of ±4.8 per cent.

In another smaller series of eleven urines the results of turbidity titrations with other alkyldimethylbenzylammonium compounds were compared with results of the biuret reaction as applied by Hiller, Greif, and Beckman (5).

For myristamidopropyldimethylbenzylammonium chloride the mean was 100.9 per cent of the biuret results, with an estimated standard deviation of ±4.45 per cent; for cetyldimethylbenzylammonium chloride the mean was 100.6 per cent of the biuret results, with an estimated standard deviation of ±3.65 per cent; for dodecyldimethylbenzylammonium chloride the mean was 99.6 per cent of the biuret results, with an estimated standard deviation of ±4.59 per cent; and for Zephiran the mean was 100.9 per cent of the biuret results, with an estimated standard deviation of ±5.56.

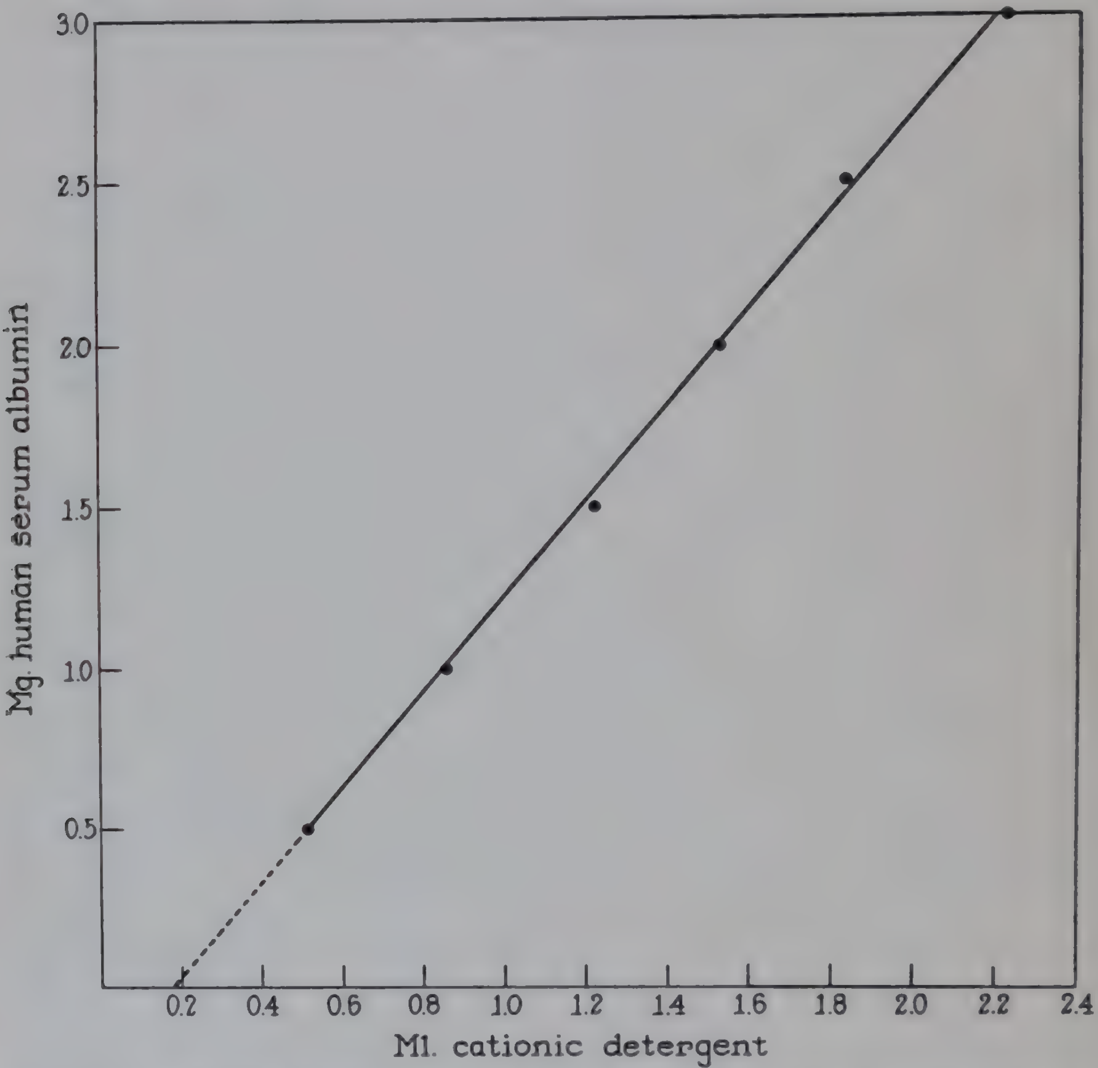


Fig. 1. "Standard curve" of human serum albumin titrated with myristamidopropyldimethylbenzylammonium chloride (1:500).

Relation of Optical Density to Amounts of Detergent Added to Solutions of Albumin—Fig. 3 shows the result of adding increasing amounts of detergent solution to 0.2, 0.5, 1.0, and 1.5 mg. of human serum albumin under the conditions described under "Procedure." The solutions were not made up to the same final volume, as it was desired to check the end-point used under the conditions of the estimations. The optical densities were measured in cylindrical cuvettes (12 × 75 mm. outside diameter) in a Coleman model 6 clinical spectrophotometer at $\lambda = 450$ mμ, within about 30 minutes of the addition of detergent to the first of a series. It will be seen that if an optical density of 0.025 is taken as the end-point (this is the approximate optical density used as the visual end-point for the data in Figs. 1 and 2) the number of ml. of detergent added to the sample containing the smallest amount of protein is not in proportion to the number of ml. added to

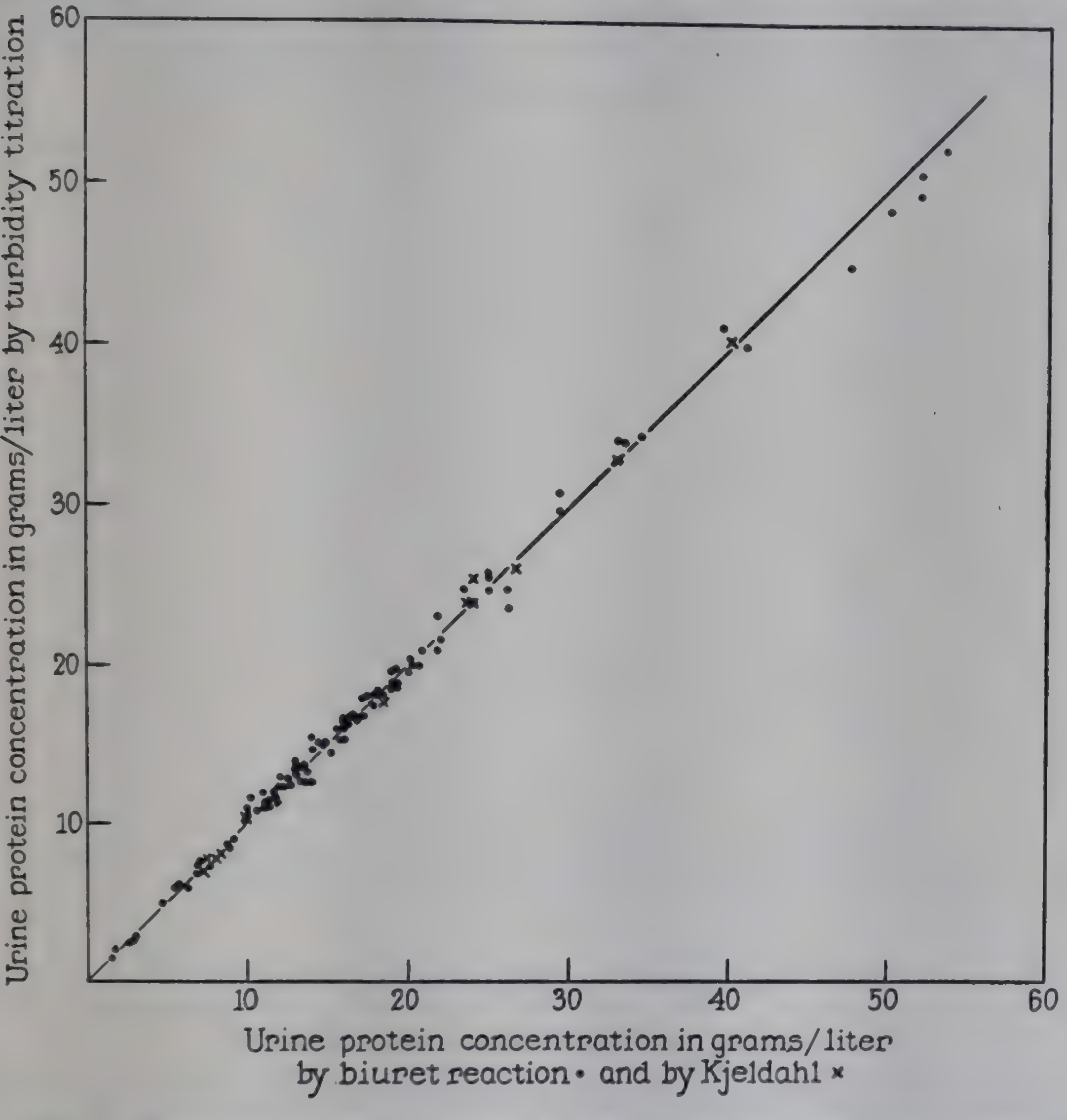


Fig. 2. Comparison of results obtained by turbidity titration with myristamidopropyldimethylbenzylammonium chloride (1:500) by the biuret reaction and the Kjeldahl method.

samples containing larger amounts of proteins. Therefore, if protein were calculated as directly proportional to the detergent added, one would somewhat overestimate the amount of protein present, especially for very small amounts of protein. That the estimated "equivalence" end-points are in reasonably good proportion (0.13, 0.30, 0.60, 0.90 ml. of detergent for 0.2, 0.5, 1.0, and 1.5 mg. of protein, respectively), however, confirms the stoichiometry of the procedure already demonstrated in Fig. 1.

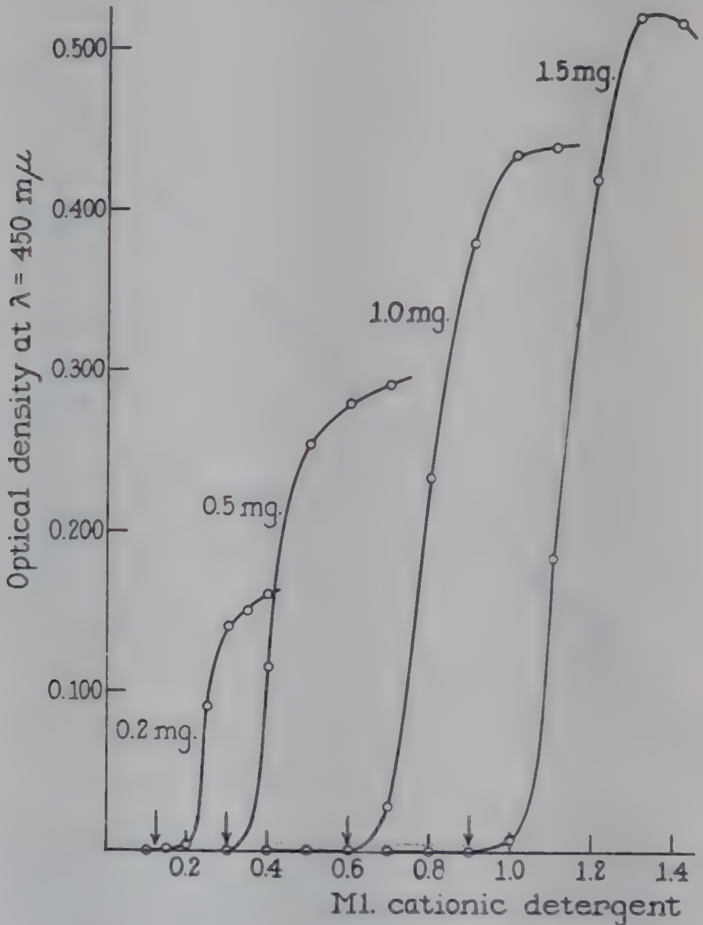


Fig. 3. Relation of optical density to amounts of detergent (myristamidopropyldimethylbenzylammonium chloride) added to solutions of human serum albumin. The arrows indicate the estimated "equivalence" end-points.

Effect of pH—Fig. 4 shows the optical density curves obtained by adding varying amounts of detergent to the same amount of protein, namely 1.0 mg. of human serum albumin, at pH 7.0, pH 7.8, pH 9.2, respectively, under the conditions described under "Procedure" (pH approximately 13). Addition of 1 ml. of 2 N NaOH instead of 0.2 ml. results in a very slight shift of the curve to the right.

Other Detergents—A few other cationic detergents have been tried, as mentioned in foot-note 1. Dimethylbenzylcetylammonium chloride, dimethylbenzyldodecylammonium chloride, and Zephiran give results similar to those obtained with the detergent routinely used as described above. The high molecular weight alkylimidazolinium compounds are not satisfactory for the present purposes because of the slowness of the development of the turbidity. Alkyltrimethylammonium compounds give indefinite end-points. Unsatisfactory results were also obtained with an N-(acylcolaminoformylmethyl)pyridinium chloride, and with cetylpyridinium chloride.

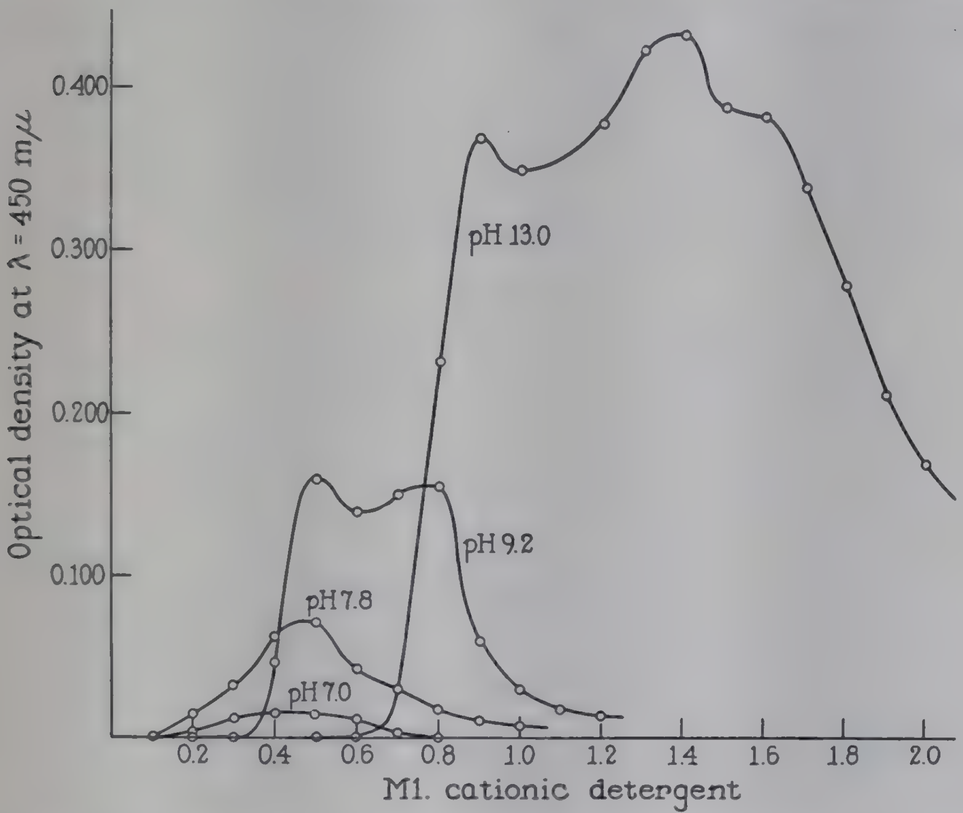


Fig. 4. Effect of pH on titration curves of human serum albumin with myristamidopropyldimethylbenzylammonium chloride.

Relation of Optical Density to Amount of Detergent Added to Plasmas, with Differing Albumin and Globulin Concentrations—The method is not at present recommended for plasma proteins, because they present some difficulties not encountered in urine. Because of the turbidity present before titration in some plasmas it is not possible in all cases to use the visual end-point described above to estimate total protein in plasma. In some preliminary experiments with plasmas of varying albumin and globulin concentrations the optical density curves show good correspondence between ml. of detergent added to reach the "equivalence" end-point and total protein present. Peculiarities appear in the titration curves in the presence of abnormally large amounts of γ-globulins. Further work on the plasma proteins is under way.

DISCUSSION

While a considerable amount of work has been done on anionic and cationic detergent-protein complex formation, the exact nature of the process is not completely known. It is apparent from the work of Putnam and Neurath (6) that the cationic groups of proteins play a major rôle with anionic detergents at acid pH. Similarly at alkaline pH the negative charges of the protein molecules must play a major rôle with cationic detergents. It may be noted, however, that the detergents are protein denaturants (7) and under certain conditions act to accelerate protein hydrolysis (8). Further, there is probably an unfolding of certain proteins in the presence of increasing amounts of detergents; native proteins cannot always be recovered quantitatively after exposure to the detergents (6, 9).

For the present work, a tentative working hypothesis (an extension of that of Polonovski and Macheboeuf (1)) is as follows: At pH 13, the carboxyl, phenolic, and sulfhydryl groups are all dissociated; there are still some positively charged guanidine groups, but the charges of the protein molecule are mainly negative. As detergent is added to the protein solution, the positively charged quaternary ammonium ions are attracted to the negative charges of the protein molecule by Coulomb forces, van der Waals forces playing a rôle dependent on the substituents of the nitrogen of the detergent. As more and more of the negative charges of the protein are "neutralized" by the positively charged quaternary ammonium ions, the protein molecule becomes less and less polar until finally, at the so called "equivalence" end-point, aggregation of the protein molecules becomes possible through van der Waals forces. With further addition of detergent the non-polar portions of the added detergent become associated through van der Waals forces with the non-polar portions of the detergent ions attached to the protein, the protein-detergent complexes become positively charged and repel each other, and dispersal of the aggregates then takes place. The N substituents in the detergent ions play a rôle in determining the extent of the van der Waals forces and may be responsible for the unsatisfactory end-points obtained with the alkyltrimethyl, alkyldimethylethyl, alkylimidazolinium, and alkylpyridinium quaternary compounds. With the alkyldimethylbenzyl compounds the number of moles of detergent required to reach the "equivalence" end-point (Fig. 3) per mole of human serum albumin varies with the detergent used, though it is of the order of magnitude of the sum of the total free carboxyl, phenolic, and sulfhydryl groups calculated from Brand's data (10). Chain length of the alkyl group may be of importance in this. A similarity to the zone phenomenon of antigen-antibody titration is evident in the fact that a definite protein-detergent ratio is required to produce aggregation.

In any case, whatever the exact nature of the protein-detergent complex, it is evident that reasonably quantitative results are obtainable by the procedure herein described. A method, to which the present one is similar in principle, was recently introduced by Lambert (11) for the volumetric analysis of anionic and cationic detergents by turbidity titration. Maximum turbidity measured photometrically is used by Lambert as the end-point, rather than the appearance of a definite turbidity estimated visually. Use of maximum turbidity is possible for the estimation of proteins, but the additional equipment required and the extra time and manipulations involved would reduce the advantages of the present procedure. The technique described by Lambert takes about 5 times as long.

SUMMARY

A rapid simple method for the estimation of urinary protein concentrations is presented. The method depends on the formation of an insoluble complex between quaternary ammonium ions and proteins at pH 13. End-points are estimated visually by the appearance of a faint but definite and permanent turbidity. The standard deviation was ±3.71 per cent from determinations by the Kjeldahl method in a series of twelve estimations. In a series of 105 estimations, the standard deviation was ±4.8 per cent from determinations by the biuret reaction. Several types of quaternary ammonium compounds have been tried; the most satisfactory for the present purposes are the alkyldimethylbenzylammonium group. A brief discussion of the possible mechanism of the quaternary ammonium ion-protein anion complex formation is given.

It is a pleasure to acknowledge the help given by Dr. Hiller and Dr. Greif in making available their biuret procedure before its publication.

BIBLIOGRAPHY

1. Polonovski, J., and Macheboeuf, M., *Ann. Inst. Pasteur*, **74**, 196, 203 (1948).
2. Schmidt, K. H., *Z. physiol. Chem.*, **277**, 177 (1942).
3. Valko, E. I., *Ann. New York Acad. Sc.*, **46**, 451 (1946).
4. Hiller, A., Plazin, J., and Van Slyke, D. D., *J. Biol. Chem.*, **176**, 1401 (1948).
5. Hiller, A., Greif, R. L., and Beckman, W. W., *J. Biol. Chem.*, **176**, 1421 (1948).
6. Putnam, F. W., and Neurath, H., *J. Am. Chem. Soc.*, **66**, 692 (1944).
7. Anson, M. L., *J. Gen. Physiol.*, **23**, 239 (1939).
8. Steinhardt, J., and Fugitt, C. H., *J. Res. Nat. Bur. Standards*, **29**, 315 (1942).
9. Putnam, F. W., and Neurath, H., *J. Am. Chem. Soc.*, **66**, 1992 (1944).
10. Brand, E., *Ann. New York Acad. Sc.*, **47**, 187 (1946).
11. Lambert, J. M., *J. Colloid Sc.*, **2**, 479 (1947).

USE OF THE HYPOBROMITE REACTION FOR THE ESTIMATION OF AMMONIA PLUS UREA NITROGEN IN URINES CONTAINING LARGE AMOUNTS OF PROTEIN; THE REACTION OF ALKALINE HYPOBROMITE WITH PROTEINS

By FRANCIS P. CHINARD

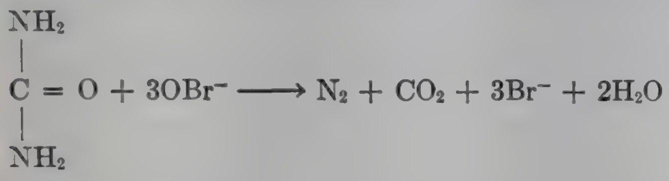
With the Technical Assistance of Dora M. Newell

(*From the Hospital of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, July 28, 1948)

Alkaline hypohalites have been in use for nearly a century for the estimation of urea or of urea and ammonia nitrogen in blood and urine. Alkaline hypochlorite, first used by Davy (1) in 1854, was replaced a few years later by alkaline hypobromite as a result of Knop's work (2). The N_2 evolved in the following reactions was measured volumetrically.

(1)



$$\mathrm{C(NH_2)_2{=}O} + 3\mathrm{OBr^-} \longrightarrow \mathrm{N_2} + \mathrm{CO_2} + 3\mathrm{Br^-} + 2\mathrm{H_2O}$$

(2)

$$2\mathrm{NH_4^+} + 4\mathrm{OBr^-} \longrightarrow \mathrm{N_2} + 4\mathrm{Br^-} + 4\mathrm{H_2O}$$

After Knop's work there was a spate of modifications of the method, modifications having to do with concentrations of the reagents, length of reaction time, design of apparatus, elimination, and occasionally identification of interfering substances. These modifications finally culminated in the manometric procedure of Van Slyke (3) as modified by Van Slyke and Kugel (4). This method has been extensively used both in this laboratory and elsewhere for the estimation of the blood and urine urea nitrogen from which, with knowledge of the urine flow, the urea clearance can be calculated.

While reasonably satisfactory when only approximate results are required, the hypobromite methods all suffer from the fact that the hypobromite reaction is neither specific nor quantitative. Many other substances besides urea and ammonia evolve nitrogen; some, such as guanidine and mono-substituted guanidines, give off nitrogen in considerable amounts; others, such as amino acids, amines, and peptides, in smaller amounts. Proteins evolve N in amounts determined chiefly by their arginine content. In addition, glucose in the high concentrations occasionally present in the urine of diabetics can cause error (5, 6).

Under the conditions of blood or plasma analyses, the hypobromite liberates approximately 98 per cent of the urea nitrogen; under the conditions of the urine analyses, hypobromite liberates approximately 95 per cent of the urea and ammonia nitrogen (3, 4). In the case of blood or plasma, the presence of non-urea N-evolving substances in the filtrates requires that a subtractive correction be applied to the calculated results; in the case of urine, one depends on the evolution of nitrogen from non-urea or ammonia substances to compensate approximately for the deficit of 5 per cent in the nitrogen evolved from the urea and ammonia (3). In some cases, however, and especially when protein is present, the evolution of nitrogen instead of approximately compensating may introduce a positive error as high as 30 per cent. While it is possible to correct approximately for the protein error if the protein concentration in the urine is known, it is preferable to remove the proteins before doing the analyses.

It is the purpose of the work reported here to present some additional data on possible sources of error inherent in the hypobromite procedure as at present applied (3, 4) and to describe an obvious means of circumventing the protein error. In addition, the reaction of alkaline hypobromite with certain proteins and compounds will be described briefly.

Removal of Proteins from Urine

The zinc hydroxide procedure of Somogyi (7) is used because it has been found to be effective in removing some of the interfering substances of blood as well as proteins (4).

Reagents

Acid zinc sulfate solution. 12.5 gm. of $ZnSO_4 \cdot 7H_2O$ are dissolved in 125 ml. of 0.25 N H_2SO_4 and diluted to 1 liter. This solution is used for the precipitation of proteins from *whole blood. For urine and plasma* 100 ml. of this solution are diluted to 212 ml. with water.

0.75 N NaOH. When 50 ml. of the zinc sulfate solution for whole blood are titrated with 0.75 N NaOH, with phenolphthalein as indicator, 6.7 to 6.8 ml. of the alkali should be required. The solution is shaken vigorously during the titration.

Procedure

For urine containing less than 80 gm. of protein per liter, 1 ml. of urine is pipetted into a centrifuge tube, and 8.5 ml. of the zinc sulfate diluted for urine or plasma are added, followed by 0.5 ml. of the 0.75 N NaOH. The tube is stoppered, shaken vigorously, and allowed to stand for 10 minutes. It is then centrifuged for 10 minutes at 2000 R.P.M. or more (18 cm. radius). The supernatant solution is then filtered through a pledget of washed cotton placed in the stem of a funnel, and aliquots are taken for analysis. A blank analysis is run on 0.9 per cent NaCl to correct for the non-urea nitrogen-liberating substances in the cotton and the reagents.

For urine containing 80 or more gm. of protein per liter, 0.5 ml. samples of urine are taken, and 0.5 ml. of distilled water is added to each. The diluted urines are then treated as above.

Details concerning manometric determination of urea in the filtrates and the factors used in calculations are given in the original publications (3, 4).

TABLE I

Comparison of Nitrogen Evolved from Urines Containing Protein and Protein-Free Filtrates of Same Urines

Urine No.	N before protein removal (a)	N after protein removal (b)	(a) − (b)	(a) as per cent of (b)	Protein concentration
	gm. per l.	gm. per l.	gm. per l.		gm. per l.
1	2.338	1.908	0.430	122.6	57.5
2	0.734	0.606	0.128	121.1	17.3
3	2.038	1.574	0.464	129.6	59.5
4	2.250	1.908	0.342	118.0	35.0
5	6.050	5.710	0.340	106.0	35.0
6	5.340	5.162	0.178	103.4	28.0
7	0.621	0.487	0.134	127.6	15.0
8	2.568	2.481	0.087	103.6	12.0
9	5.635	5.371	0.264	104.9	36.0
10	7.850	7.468	0.382	105.1	60.5
11	4.829	4.680	0.139	103.2	18.0
12	1.965	1.882	0.083	104.4	6.7
13	7.100	6.780	0.320	104.7	39.5
14	2.937	2.858	0.079	102.8	3.6
15	7.278	7.175	0.103	101.4	4.6
16	5.418	5.198	0.220	104.2	18.0
17	2.803	2.698	0.105	103.9	10.4
18	2.051	1.915	0.146	107.1	38.6
19	4.714	4.554	0.160	103.5	13.0
20	7.108	6.823	0.185	104.1	27.9

Comparison of N Evolved from Urine Containing Protein and from Protein-Free Filtrates of Same Urines

Table I shows the differences in N evolved from urines containing protein and protein-free filtrates of the same urines. The urines were obtained from patients with the nephrotic syndrome, some of whom were receiving large doses of human serum albumin intravenously. Generally speaking, unless the urines are from patients receiving serum albumin or plasma intravenously, urine protein concentrations will not exceed 25 to 30 gm. per liter, provided the urine flow is reasonably high (1 or more ml. per minute). If, however, the patient's urine flow is small (less than 1 ml. per minute), then the urine protein concentration may rise to 40 or 50 gm. per liter. The urine protein concentrations were calculated from nitrogen determinations done by the micro- or macro-Kjeldahl procedures of Hiller, Plazin, and Van Slyke (8). The total nitrogen was determined directly, the non-protein nitrogen was determined after removal of the proteins by precipitation with equal volumes of 10 per cent trichloroacetic acid, and the protein nitrogen was calculated from the difference. It will be noted that the errors due to protein are all positive and vary from +1.4 per cent to +29.6 per cent. The magnitude of the percentage error is determined by the ratio of the protein N evolved by hypobromite to the N evolved from the urea plus ammonia, not by the absolute amount of protein present. Fig. 1, in which protein concentration is plotted against the difference in N evolved before and after removal of the proteins, shows that there is considerable scattering. A correction made on the basis of the data of Table I would be approximate, though adequate for clinical purposes. Fig. 1 is to be compared with Fig. 2 which shows, from analyses of pure

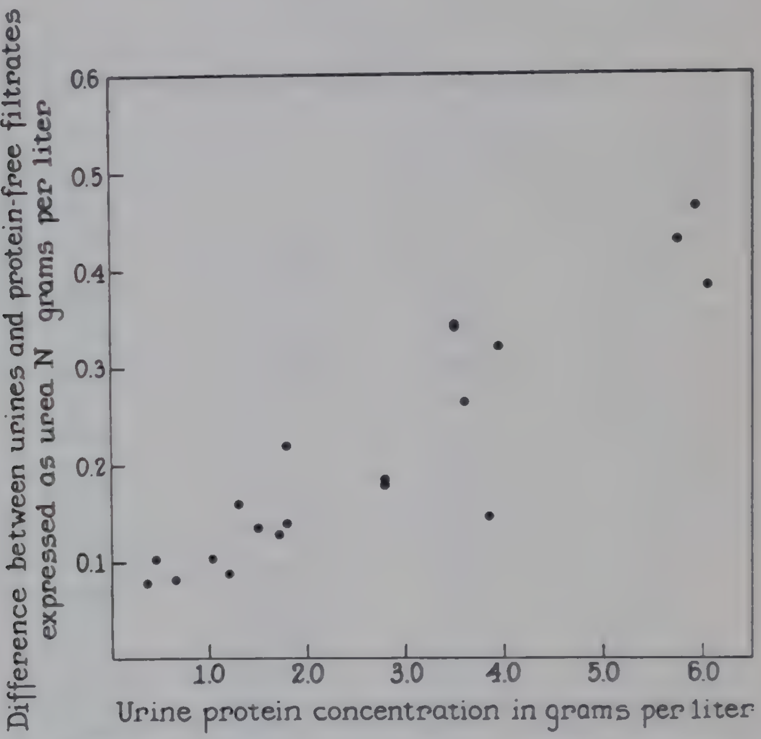


Fig. 1. Relationship of concentration of urine protein to difference in N evolved from urines and protein-free filtrates.

albumin solutions, the relatively close proportionality of N evolved to albumin present under the conditions of routine urine analyses when other N-evolving substances are absent. An additional point is that, in protein-free urine from normal individuals, treatment of the urine with the Somogyi reagents resulted in lower values for urea plus ammonia N than in untreated urine; this effect is presumably due to precipitation of interfering substances other than protein.

It may be noted here that the reaction of alkaline hypobromite with proteins and amino acids is not restricted to the guanidino group of the arginine; tyrosine is brominated, free amino groups react, and there is also reaction with peptide groups (see, for example, Goldschmidt *et al.* (9, 10)). These reactions occur with little or no evolution of N. The result is that much more hypobromite is used up in reaction with proteins than would be expected from the N liberated. This fact becomes of importance when the protein concentrations are very high, because the reaction with protein may not leave enough hypobromite to give the expected N yield from urea. This effect has been found in a few cases in which the urine was not sufficiently diluted before analysis; less N was evolved per aliquot of urine than after greater dilution. If protein-free filtrates are used, error from this effect of excess protein is avoided.

Evolution of N from Various Proteins

The evolution of N from human serum albumin was studied in some detail. Under the conditions of the routine urine analyses (3, 4) (2.5 minutes reaction time measured from the moment of addition of the alkaline hypobromite to the moment the solution was brought to the 2.0 ml. mark of the gas chamber for reading the volume), a reasonably stoichiometric relationship was found between mg. of N evolved and mg. of protein present, as illustrated in Fig. 2. It was found, however, that if the reaction time was prolonged beyond 2.5 minutes more nitrogen was evolved, though at a much slower rate than during the first 2.5 minutes. An example of the time course of the reaction is given in Fig. 3 for 20.0 mg. of human serum albumin (Curve A), and for 18.35 mg. of bovine γ-globulin (Curve B). Similar curves were obtained with edestin and gelatin.

Because arginine is the only guanidine derivative reported in proteins in appreciable amounts, it was thought that the N evolved from intact proteins by alkaline hypobromite might give a measure of the arginine content of those proteins. It was found, however, in the few proteins examined that less N was evolved than was calculated from the arginine contents. While the results were quite reproducible provided the reaction times were the same, in no case was the calculated amount of N evolved. The data in Table II were obtained from proteins in aqueous solutions of

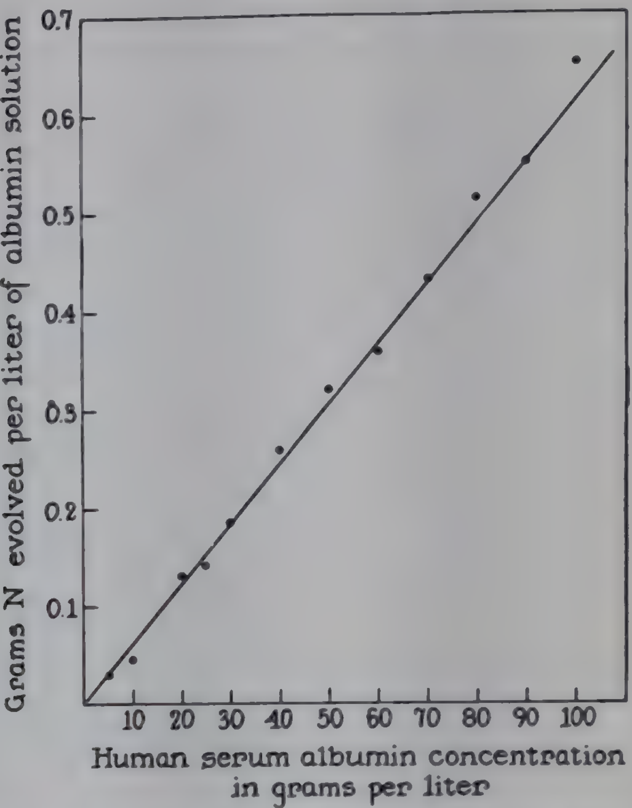


FIG. 2. Evolution of N from various amounts of human serum albumin in 2.5 minutes.

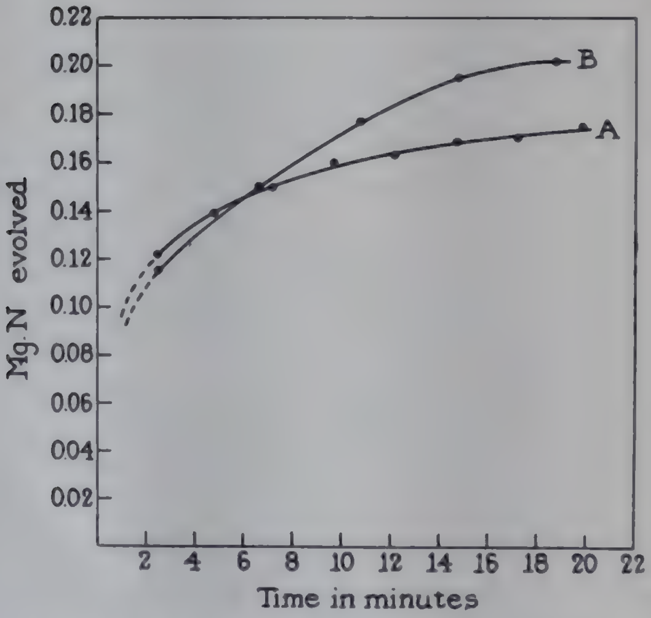


FIG. 3. Evolution of N from 20.0 mg. of human serum albumin (Curve A) and from 18.36 mg. of bovine γ-globulin (Curve B).

approximately 1 per cent concentration, and the results were calculated on the basis of total protein N as determined by macro-Kjeldahl analysis (8). The values for arginine N content were taken from tables published by Chibnall (11) and Brand (12) and were not determined by analysis of the protein preparations used in these experiments; for this reason the data should be considered as preliminary.

TABLE II

Evolution of Nitrogen from Various Proteins by Alkaline Hypobromite

Protein	Protein N in sample (*a*)	Calculated arginine N in sample (*b*)	Calculated N evolvable by hypobromite* (*c*)	N evolved by hypobromite in 2.5 min. (*d*)	N evolved as per cent of (*c*)
	mg.	*mg.*	*mg.*	*mg.*	
Human serum albumin......	3.190	0.3990	0.1894	0.1220	64.4
Gelatin....................	3.402	0.5175	0.2459	0.2373	92.9
Edestin....................	3.404	0.9820	0.4661	0.3745	80.3

* Calculated N corrected for 5 per cent deficit in N evolved from urea and arginine under the conditions used for the analysis. Calculated arginine N is based on data of Brand (12) for human serum albumin. For gelatin and edestin, calculations are based on data assembled by Chibnall (11).

Evolution of N by Hypobromite Reaction from Guanidine, Guanidine Derivatives, Amino Acids, and Other Compounds

Under the conditions of the routine urinalysis, many substances besides proteins were found to evolve N when they reacted with alkaline hypobromite. In particular, guanidine and its derivatives of the type R_1R_2-N—C(=NH)—NH_2 (where R_1 and R_2 may be H, alkyl, or aryl groups) give off nearly two-thirds of their guanidine nitrogen. Of such a type are, for example, arginine, methylguanidine, creatine, and guanidoacetic acid, and of a similar type are dicyandiamide and guanylurea. The evolution of N from the guanidino groups is quite rapid with these compounds and is complete or nearly complete in the 2.5 minutes required for urine analysis. Another group of substances, aliphatic diamines, evolve N at a slower but still appreciable rate. Pentamethylene- and hexamethylenediamine, ornithine and lysine evolve about 3 per cent of their total nitrogen in 2.5 minutes, and about 10 per cent of their total nitrogen in 10 minutes. This evolution of nitrogen may stem from nitrile formation, ring closure to form an amidine, and reaction of this latter group with more hypobromite. In contrast, monoamino-, mono-, or dicarboxylic acids, asparagine, glutamine, and creatine evolve practically no N in the routine reaction time. Glutathione evolves slightly less N per mole than do the diamines. Mono-N-substituted ureas evolve nearly one-half their total urea nitrogen.

In addition to the above nitrogen compounds, glucose also evolves gas which is measured as N in the routine analyses. A 1 per cent solution of glucose evolves an amount of gas equivalent to approximately 0.024 gm. of N per liter. Hence the hypobromite method should not be employed for analyses of urine from diabetics.

DISCUSSION

The sources of error and their significance are apparent from the above paragraphs. Of ancillary interest is the finding that not all of the arginine guanidino groups in proteins appear to be available for reaction with alkaline hypobromite under the condition used; this suggests that some of these groups may be involved in linkages and are therefore not free. (There is little likelihood that this effect is due to exhaustion of the OBr^- because of the proportionality of the results with different amounts of protein. The expected N is evolved from protein-urea mixtures.) Similar observations have been made in the case of egg albumin by Goldschmidt *et al.* (9); in this protein even after a 4 hour exposure to hypobromite, there was still some intact arginine which could be demonstrated, suggesting that the arginine linkages in proteins were not all identical and that some of the guanidino groups were protected. Of similar import are the observations of Roche and his collaborators (see, for example, (13)) who have used the Sakaguchi reaction on intact proteins and found less color developed than would be expected from the total arginine content of the proteins examined. In addition, Simms has suggested, from the evidence offered by his titration curves of certain proteins, that some of the arginine guanidino groups are somehow linked to other portions of the protein molecule (14). Further work is under way at present on the reaction of hypobromite with proteins and guanidine derivatives; preliminary experiments suggest that the hypobromite reaction may be of some use for rapid semimicroestimation of arginine in protein hydrolysates (*cf.* (15)).

SUMMARY

Under the conditions used for gasometric determination of urea and ammonia by the hypobromite reaction, the greater part of the guanidino groups in proteins reacts with evolution of nitrogen gas. In urine with high protein concentration (*e.g.* 30 or more gm. per liter) the nitrogen gas evolved from the proteins may cause a plus error of as much as 30 per cent in the urea determination. This error can be prevented by preliminary removal of the proteins by Somogyi's zinc hydroxide precipitation.

The reaction of hypobromite with human serum albumin, bovine γ-

globulin, edestin, and gelatin has been studied with regard to its time course and evolution of total nitrogen gas. The final amounts of nitrogen gas evolved were from 64 to 93 per cent of that which would be evolved by the guanidino groups of arginine in the amounts reported to be present in these proteins. It appears that some of the guanidino groups in the protein molecules are not free to react with alkaline hypobromite.

The reactions of other substances with hypobromite have been reviewed. Glucose, if present in more than 2 per cent concentration, will cause evolution of enough gas to produce a significant positive error in determination of urine urea.

BIBLIOGRAPHY

1. Davy, E. W., *Phil. Mag.*, **7**, 385 (1854).
2. Knop, W., and Wolf, W., *Chem. Zent.*, **5**, 257 (1860).
3. Van Slyke, D. D., *J. Biol. Chem.*, **83**, 449 (1929).
4. Van Slyke, D. D., and Kugel, V. H., *J. Biol. Chem.*, **102**, 489 (1933).
5. Esbach, G., *Compt. rend. Acad.*, **89**, 417 (1879).
6. Krogh, M., *Z. physiol. Chem.*, **84**, 379 (1913).
7. Somogyi, M., *J. Biol. Chem.*, **86**, 655 (1930).
8. Hiller, A., Plazin, J., and Van Slyke, D. D., *J. Biol. Chem.*, **176**, 1401 (1948).
9. Goldschmidt, S., Wolff, R. R., Engel, L., and Gerisch, E., *Z. physiol. Chem.*, **189**, 193 (1930).
10. Goldschmidt, S., Wiberg, E., Nagel, F., and Martin, R., *Ann. Chem.*, **456**, 1 (1927).
11. Chibnall, A. C., *J. Internat. Soc. Leather Trades' Chem.*, **30**, 1 (1946).
12. Brand, E., *Ann. New York Acad. Sc.*, **47**, 187 (1946).
13. Roche, J., and Blanc-Jean, G., *Compt. rend. Acad.*, **210**, 681 (1940).
14. Simms, H. S., *J. Gen. Physiol.*, **14**, 87 (1930).
15. Tsuverkalov, D. A., *Biokhimyia*, **9**, 101 (1944).

LETTERS TO THE EDITORS

THE USE OF LACTOBACILLUS LEICHMANNII IN THE MICROBIOLOGICAL ASSAY OF THE "ANIMAL PROTEIN FACTOR"

Sirs:

During the course of isolation studies on the "animal protein factor" we have employed a microbiological assay method to follow its separation and purification from various naturally occurring crude materials. Specific data showing the excellent correlation obtained between the microbiological and mouse growth[1] assay methods for the "animal protein factor" will be published at a later date.

Concentrate per tube	0.1 N acid produced
γ	*ml.*
0	3.20
0	3.30
0.005	4.45
0.010	5.65
0.015	6.80
0.020	8.65
0.030	10.50
0.050	11.40

Medium: The 100 ml. double strength medium contains 1.0 gm. of acid-hydrolyzed norit-treated casein (*Univ. Texas Pub., No. 4137*, 82 (1941)); 20 mg. each of tryptophan and cystine; 1 mg. each of adenine, guanine, xanthine, and uracil; 1 ml. each of Salts A, Salts B (Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941)); 1.2 gm. of sodium acetate; 4 gm. of glucose; 1 γ of biotin; 400 γ of pyridoxine; 400 γ of pyridoxal; 200 γ each of riboflavin, thiamine, pantothenic acid, nicotinic acid; 100 γ of folic acid; 0.2 ml. of Tween 80 and 500 mg. of norit-treated tryptic digest of casein. (The casein digest is prepared as follows: 25 gm. of Labco casein are suspended in 250 ml. of 0.8 per cent $NaHCO_3$ and incubated under benzene with 0.5 gm. of trypsin for 48 hours. After digestion, the material is autoclaved 15 minutes and filtered. The filtrate is taken to pH 2.0, stirred 1 hour with 10 gm. of norit A, and filtered. The pH of the filtrate is then adjusted to 6.6 to 6.8.) *Inoculum:* 0.1 ml. of 24 hour culture of *L. leichmannii* grown in 1 per cent tryptose milk tube suspended in 10 ml. of saline. *Sterilization:* Autoclave 15 minutes at 120°. *Incubation:* 37° for 24 hours for turbidimetric determination or 72 hours for titrimetric determination.

---

[1] Bosshardt, D. K., Paul, W. K., O'Doherty, K., Huff, J. W., and Barnes, R. H., Abstracts, American Chemical Society, 113th meeting, 22C (1948).

The organism employed in the microbiological assay is *Lactobacillus leichmannii* (ATCC 4797). Its response to a highly purified preparation, prepared in these laboratories, demonstrated to have "animal protein factor" activity in the mouse, is shown in the table. Using the basal medium and the conditions described in the table, we routinely have obtained highly satisfactory assay results with *L. leichmannii* for over a year.

Recent reports from the Merck laboratories[2] have indicated that the "animal protein factor" is identical with or closely related to vitamin B_{12}. If such is the case, it is probable that *L. leichmannii* may be the organism of choice for the microbiological assay of vitamin B_{12}. The complex growth requirements of *L. lactis* (ATCC 8000), currently employed as the assay organism for vitamin B_{12},[3] have made its use in routine procedures difficult and unpredictable. We have found that both organisms will respond to purified liver preparations (shown to be active as "animal protein factor" in mice) and similarly to relatively large amounts of thymidine.[4] The effects of ascorbic acid and air, as reported by Shive *et al.*[5] for *L. lactis*, have been demonstrated with *L. leichmannii*. Autoclaving the tests for 15 minutes at 120° minimized the effects of ascorbic acid and air, but the growth stimulation by thymidine must be considered in the interpretation of results, particularly when crude materials are assayed.

Department of Nutrition and Biochemistry
Medical Research Division
Sharp and Dohme, Inc.
Glenolden, Pennsylvania

Helen R. Skeggs
Jesse W. Huff
Lemuel D. Wright
David K. Bosshardt

Received for publication, August 25, 1948

---

[2] Ott, W. H., Rickes, E. L., and Wood, T. R., *J. Biol. Chem.*, **174**, 1047 (1948).

[3] Short, M. S., *Science*, **107**, 396 (1948).

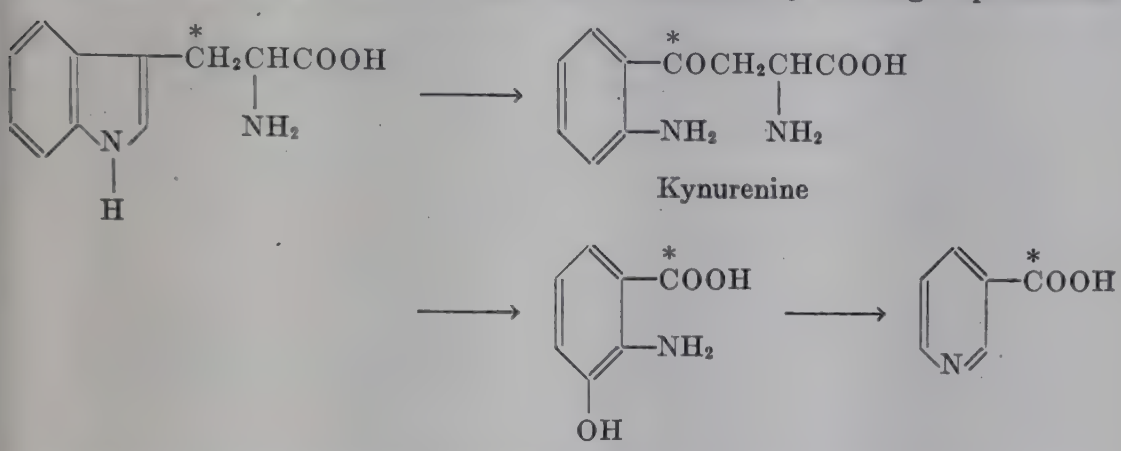
[4] Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, in press.

[5] Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Chem. Soc.*, **70**, 2614 (1948).

CONCERNING THE MECHANISM OF THE MAMMALIAN CONVERSION OF TRYPTOPHAN INTO NICOTINIC ACID[*]

Sirs:

The mechanism of the conversion of tryptophan to nicotinic acid involving kynurenine and 3-hydroxyanthranilic acid as intermediates, has been demonstrated in *Neurospora*,[1] and confirmatory feeding experiments in rats have been reported recently.[2] We have shown[3] that tryptophan-β-C^{14} is directly converted into kynurenine in the intact mammal, but, because the labeled carbon atom was lost, it was necessary (by other means) to establish its further conversion to nicotinic acid.



Compound	Specific activity, counts per min. per mg.
Tryptophan (fed)	2740
N-Methylnicotinamide picrate (carrier)	0.75
Nicotinic acid	2.0 (2.2 calculated)
Barium carbonate from decarboxylation	1.3 (1.2 “)

We have now synthesized[4] DL-tryptophan-3-C^{14}, and have shown that the conversion proceeds in the same fashion in the rat as in *Neurospora.*

---

[*] This paper is based on work performed under contract No. W-7405-eng-48 with the Atomic Energy Commission in connection with the Radiation Laboratory, University of California, Berkeley. We are also indebted to the Rockefeller Foundation for support.

[1] Mitchell, H. K., and Nyc, J. F., *Proc. Nat. Acad. Sc.*, **34,** 1 (1948).

[2] Albert, P. W., Scheer, B. T., and Deuel, H. J., Jr., *J. Biol. Chem.*, **175,** 479 (1948).

[3] Heidelberger, C., Gullberg, M. E., Morgan, A. F., and Lepkovsky, S., *J. Biol. Chem.*, **175,** 471 (1948).

[4] Heidelberger, C., unpublished material.

The tryptophan (226 mg.) was fed to three rats by stomach tube, and the 24 hour urines were passed through a permutit column. The N-methylnicotinamide (750 γ) was eluted with KCl,[5] 76 mg. of carrier N-methylnicotinamide chloride were added, and the picrate was formed and purified. The amide was freed of picric acid and converted with HCl in a sealed tube into nicotinic acid, which was purified and decarboxylated catalytically. The specific activities are shown in the table. A significant observation is that the carbon atom, which is a precursor to the carboxyl group of the hydroxyanthranilic acid, becomes the carboxyl carbon of nicotinic acid.

Department of Chemistry and the Radiation Laboratory and the Division of Poultry Husbandry
University of California
Berkeley

CHARLES HEIDELBERGER[6]
EDWARD P. ABRAHAM[7]
SAMUEL LEPKOVSKY

Received for publication, September 24, 1948

---

[5] Hochberg, M., Melnick, D., and Oser, B. L., *J. Biol. Chem.*, **158**, 265 (1945).

[6] Present address, McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison 6, Wisconsin.

[7] On leave from the Sir William Dunn School of Pathology, University of Oxford, England.

THE EFFECT OF DISSOCIATION IN LACTOBACILLUS LACTIS CULTURES ON THE REQUIREMENT FOR VITAMIN B_{12}*

Sirs:

Although *Lactobacillus lactis* Dorner (American Type Culture Collection No. 8000) is stable in its requirement for both vitamin B_{12} (LLD factor) and the tomato juice (TJ) factor when cultured on a tomato juice-yeast extract-skim milk medium, the culture undergoes dissociation[1] when serial transfers are made on the usual yeast extract-glucose agar, with or without added tomato juice. Variants produced on this stock agar may grow in the amino acid assay medium with the tomato juice supplement only, and one isolated variant required no supplement when the concentration of the amino acids was doubled. L-Histidine was responsible for a large amount of this growth.

The medium used in the assay of the LLD factor[2,3] and in connection with the isolation of vitamin B_{12}[4] is that of Baumgarten *et al.*,[5] modified by the addition of all the B vitamins at 10-fold concentration, except biotin and pteroylglutamic acid, which are used at 0.05 γ per 10 ml. Adenine, guanine, uracil, and xanthine are added at 0.1 mg., and 0.5 ml. of clarified tomato juice (TJ factor) is used routinely. An extremely small inoculum of twice washed culture is used.

The assay as outlined above works well with the stabilized culture and purified liver extracts or crystalline vitamin B_{12}, but erratic results may be obtained with the dissociating culture or with some crude materials because of the presence of inhibitory substances.[2,6] The inhibition has been found to be due, in part, to high concentrations of folic acid. High levels of serine, *p*-aminobenzoic acid, xanthine, $MnSO_4$, NaCl, and $FeSO_4$, under certain conditions, also inhibit growth.

Culture filtrates of *Lactobacillus casei*, *Streptococcus faecalis* R, and

---

* Scientific paper No. A214. Contribution No. 2134 of the Maryland Agricultural Experiment Station (Department of Poultry Husbandry). This study was supported in part by a grant from Merck and Company, Inc., Rahway, New Jersey.

[1] Braun, W., *Bact. Rev.*, **11,** 101 (1947).

[2] Shorb, M. S., *J. Biol. Chem.*, **169,** 455 (1947).

[3] Shorb, M. S., *Science*, **107,** 397 (1948).

[4] Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, **107,** 396 (1948).

[5] Baumgarten, W., Garey, J. C., Olsen, M. J., Stone, L., and Boruff, C. S., *J. Am. Chem. Soc.*, **66,** 1607 (1944).

[6] Shorb, M. S., *J. Bact.*, **53,** 669 (1947).

Escherichia coli and fresh chicken droppings (frozen for 24 hours) have a low vitamin B_{12} activity.

Department of Poultry Husbandry
University of Maryland
College Park, Maryland

MARY S. SHORB
G. M. BRIGGS[7]

Received for publication, September 24, 1948

---

[7] Present address, Division of Poultry Husbandry, University of Minnesota, St. Paul, Minnesota.

RESPONSE OF LACTOBACILLUS LEICHMANNII 313 TO THE ANTIPERNICIOUS ANEMIA FACTOR

Sirs:

Recent observations [1,2] have shown that thymidine has a growth-stimulating effect upon *Lactobacillus lactis* Dorner under conditions in which growth is also promoted by vitamin B_{12}.[3,4] Thymidine has also been found to promote the growth of *Lactobacillus leichmannii* 313.[5] Accordingly, the effect of a sample of crystalline antipernicious anemia factor (APA factor) was tested with this organism, with a basal medium similar to that described by Snell and coworkers.[5] A final volume of 2 or 4 ml. and an incubation temperature of 37° were used. Readings were made at 14 hours with the results given in the table.

Addition per ml. basal medium		Optical density
	mγ	
None		0.06
APA factor	0.0005	0.04
	0.0015	0.06
	0.005	0.12
	0.015	0.20
	0.05	0.37
	0.15	0.62
	0.5	0.97
	1.5	1.17
	5.0	1.17
	c. mm.	
Liver extract, injectable	0.005	0.17
	0.015	0.34
	0.05	0.60
	0.15	0.86
	0.5	1.17

In other experiments higher levels of liver extract did not produce growth in excess of the maximum growth obtained with the APA factor. Half maximum growth was obtained with 0.25 to 0.5 γ of thymidine per

---

[1] Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Chem. Soc.*, **70,** 2614 (1948).

[2] Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, **175,** 475 (1948).

[3] Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, **107,** 396 (1948).

[4] Shorb, M. S., *Science*, **107,** 397 (1948).

[5] Snell, E. E., Kitay, E., and McNutt, W. S., *J. Biol. Chem.*, **175,** 474 (1948).

ml.[6] When pteroylglutamic acid and *p*-aminobenzoic acid were omitted from the culture medium, the organism did not respond to the APA factor. Increasing the level of pteroylglutamic acid up to 50 γ per ml. of culture medium did not permit growth in the absence of the APA factor.

The results indicated that *Lactobacillus leichmannii* 313 may be a sensitive test organism for the crystalline antipernicious anemia factor, and that liver extract produced a similar response.

Lederle Laboratories Division
American Cyanamid Company
Pearl River, New York

C. E. Hoffmann
E. L. R. Stokstad
A. L. Franklin
Thomas H. Jukes

Received for publication, September 29, 1948

---

[6] Samples of thymidine were kindly furnished by Dr. W. Shive and Dr. D. W. Woolley.

THE SITE OF CONVERSION OF CAROTENE TO VITAMIN A*

Sirs:

In a recent paper[1] from this department evidence was presented that one site of conversion of carotene to vitamin A in the rat was the intestinal wall. Since the analytical method used in these experiments was the Carr-Price reaction which is not specific for vitamin A, it was felt that the qualitative identification of vitamin A in the intestinal wall of the rat would strengthen the conclusions drawn. The purpose of this paper is to present such evidence.

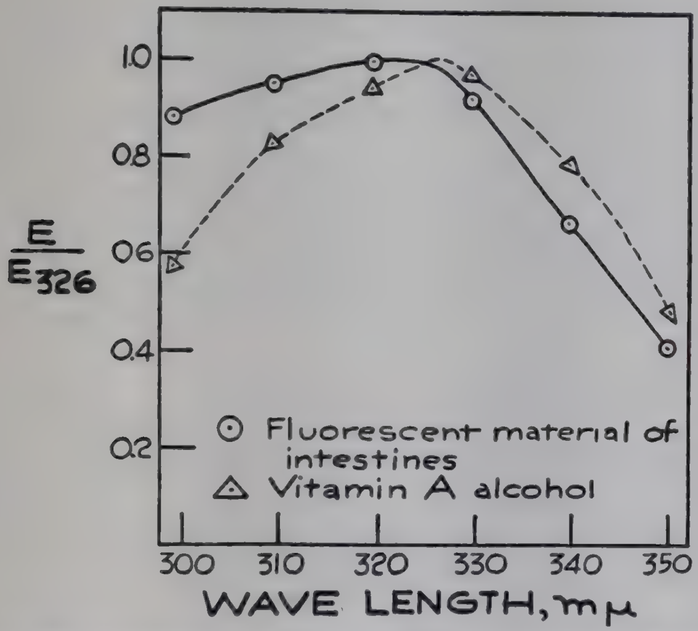


Fig. 1. The absorption curves of nitamin A alcohol and the fluorescent material of the intestinal tract.

Ten vitamin A-deficient rats were given 0.5 ml. of cottonseed oil containing 1200 γ of carotene[2] and 0.5 per cent tocopherol by stomach tube. At the end of 4 hours the animals were sacrificed and the intestinal tracts removed. This time interval was chosen, since in the previous experiments it was found that at the end of this period the vitamin A level in the intestinal wall had attained its maximum value. Although vitamin A

---

* Aided by a grant from the Nutrition Foundation, Inc. The author wishes to express his appreciation for the use of the facilities of the Hancock Foundation. Contribution No. 185 from the Department of Biochemistry, University of Southern California.

[1] Mattson, F. H., Mehl, J. W., and Deuel, H. J., Jr., *Arch. Biochem.*, **15**, 65 (1947).

[2] Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

is also present in the liver at that time, the amount in the intestinal wall exceeds that in the liver. The contents of the intestines were flushed out with 0.9 per cent sodium chloride and the intestines combined into one sample. The sample was saponified with alcoholic KOH and extracted with Skellysolve A. The petroleum ether was then extracted three times with 90 per cent methyl alcohol. In this way most of the vitamin A is extracted by the methyl alcohol, leaving the carotene in the Skellysolve. Sufficient water was then added to the methyl alcohol solution to reduce the concentration of alcohol to approximately 50 per cent. This was then extracted with Skellysolve B. The Skellysolve was dried with anhydrous sodium sulfate and reduced to a small volume in a stream of nitrogen. This was placed on a column of 1:1 magnesium oxide-Hyflo and the chromatogram developed with Skellysolve B. The fluorescing band was separated mechanically and eluted with Skellysolve B containing a small amount of ethyl alcohol. The absorption curve of this fluorescing band was determined on the Beckman spectrophotometer. These results with those determined on vitamin A alcohol[3] are shown in the graph. A mixed chromatogram of vitamin A alcohol and the fluorescent material isolated from the intestines gave a single fluorescing band on a column of magnesium oxide and Hyflo with Skellysolve B as the solvent and developing agent.

Since the material isolated from the intestines of the rats exhibited fluorescence, possessed an absorption curve similar to that of vitamin A, and showed a single fluorescing band in a mixed chromatogram with vitamin A, it is reasonably certain that vitamin A is present in the intestinal wall of rats 4 hours after supplementing with carotene.

Department of Biochemistry and Nutrition
University of Southern California School of Medicine
Los Angeles

FRED H. MATTSON[4]

Received for publication, October 4, 1948

---

[3] Obtained from Distillation Products, Inc., Rochester, New York.

[4] Present address, The Procter and Gamble Company, Cincinnati, Ohio.

OXIDATION IN VIVO OF EMULSIFIED RADIOACTIVE TRILAURIN ADMINISTERED INTRAVENOUSLY*

Sirs:

Studies conducted in this laboratory have clearly shown that fat injected intravenously in an emulsified form is utilized for energy by the dog.[1] The rate of disappearance of such fat from the blood of various species has been found to be very rapid.[2] In order to determine how rapidly such fat is metabolized *in vivo*, use has been made of an emulsion which contained radioactive trilaurin. The radioactive lauric acid present in the trilaurin contained C^{14} in the carboxyl group, and the trilaurin was made by direct esterification.[3] An emulsion was made containing 0.6 gm. of coconut oil, 0.4 gm. of the trilaurin, water, dextrose, and stabilizers, and made up to a total volume of 20 ml. This product was sealed in glass ampules under N_2 and sterilized by autoclaving.

Excretion of $C^{14}O_2$ by Rat following Intravenous Injection of Emulsified Trilaurin (—$C^{14}OO$—)

Sample No.	Time of collection		Specific activity*	Injected C^{14} activity expired
	Per sample	Total		
	min.	*min.*		*per cent*
1	10	10	4100	2.76
2	10	20	7560	7.23
3	15	35	9060	15.77
4	20	55	7910	25.39
5	35	90	7220	40.01
6	40	130	5510	51.31
7	35	165	3580	58.43
8	35	200	2640	63.78
9	35	235	2050	67.18
10	35	270	1670	70.85

* Specific activity = counts per minute per mg. of CO_2 carbon.

Although a number of metabolic studies have been carried out, only one typical example will be described here. A non-fasted female rat weighing 270 gm. was anesthetized lightly with ether and was injec intravenously through a tail vein with 3.9 ml. of the above emulsion. animal was immediately placed in a respirometer chamber and se samples of expired CO_2 were collected in 1 N NaOH. Total CO_2 det minations were made by the Van Slyke manometric technique, and rad activity assays were made on $BaCO_3$ samples prepared from the alka carbonate solution. Preparation of the samples and counting we accomplished according to the method described by Olson *et al.*[4] The resu are given in the accompanying table.

---

* Supported in part by grants-in-aid from the Nutrition Foundation, Inc., the Milbank Memorial Fund, The Upjohn Company, and the National Dairy Council.

[1] McKibbin, J. M., Ferry, R. M., Jr., and Stare, F. J., *J. Clin. Invest.*, **25,** 679 (1946).

[2] Geyer, R. P., Mann, G. V., and Stare, F. J., *J. Lab. and Clin. Med.*, **33,** 175 (1948).

[3] Details concerning the syntheses and emulsification techniques will be described in a more complete paper now in preparation.

Essentially the same results were obtained with animals fasted hours. From these data it is apparent that properly emulsified fat utilized immediately for energy following its intravenous administratio

Department of Nutrition, Harvard School of Public Health, and Department of Biological Chemistry, Harvard Medical School
Boston

Robert P. Geye
June Chipman
F. J. Stare

Received for publication, October 14, 1948

---

[4] Olson, R. E., Miller, O. N., Topper, Y. J., and Stare, F. J., *J. Biol. Chem.*, **175**, 503 (1948).

2,6-DIAMINOPURINE, A PRECURSOR OF NUCLEIC ACID GUANINE*

s:

2,6-Diaminopurine (2-aminoadenine), which was postulated[1] as one of e possible intermediates involved in the conversion of adenine into ıcleic acid guanine,[2] has been synthesized[3] with isotopic nitrogen in the 1

	Atom per cent excess N^{15}*		Calculated on basis of 100 per cent N^{15} in purine fed
2,6-Diaminopurine (dietary)	5.44	5.45	100
Sodium nucleic acids	0.076	0.075	
Copper purines	0.148	(0.157†)	
Adenine (calculated from picrate)‡	0.043		0.78
" (free)‡	0.042		
Guanine sulfate‡	0.222		4.0
" (free)‡	0.216		
Silver pyrimidines	0.022		0.40
Allantoin	0.336	0.335	6.2
Urea	0.023	0.023	0.42
Ammonia	0.020	0.022	0.38
Total urinary nitrogen	0.051	0.054	0.96

* Consolidated-Nier ratio mass spectrometer; duplicates are on independent digestions and conversions.

† Insufficient gas sample for optimum determination.

‡ The guanine was shown to be free of adenine by paper chromatography (Vischer, E., and Chargaff, E., *J. Biol. Chem.*, **176,** 703 (1948)). The adenine contained a trace of foreign material, not definitely identified as guanine.

and 3 positions of the ring and in the 2 amino group. It was administered by stomach tube to rats at a level of 29 mg. per kilo per day for 3 days.

---

* The authors wish to acknowledge the assistance of the James Foundation of New York, Inc., the National Cancer Institute of the United States Public Health Service, and the Office of Naval Research.

[1] Brown, G. B., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **13,** in press.

[2] Brown, G. B., Roll, P. M., Plentl, A. A., and Cavalieri, L. F., *J. Biol. Chem.*, **172,** 469 (1948).

[3] Bendich, A., Tinker, J. F., and Brown, G. B., *J. Am. Chem. Soc.*, **70,** 3109 (1948).

The urinary constituents and the total nucleic acids of the viscera were examined (see the table).

The results indicate that 2,6-diaminopurine is an effective precursor of nucleic acid guanine.

It is interesting that both adenine and 2,6-diaminopurine are toxic for rats at levels at which other purines have proved to be non-toxic, and that 2,6-diaminopurine evinces *apparent* "antipurine-metabolite" behavior[4] under certain conditions. In so far as the authors are aware, the presence of this purine has not been detected in nature.

Sloan-Kettering Institute for Cancer Research
New York

AARON BENDICH
GEORGE BOSWORTH BROWN

Received for publication, October 15, 1948

---

[4] Hitchings, G. H., Elion, G. B., VanderWerff, H., and Falco, E. A., *J. Biol. Chem.* **174**, 765 (1948).

GLUTAMINE AND THE GROWTH OF STAPHYLOCOCCUS AUREUS

Sirs:

Grossowicz[1] recently reported experiments from which he concluded that the addition of 0.6 to 2.0 mg. of L-glutamine per ml. of medium (supplemented casein hydrolysate) inhibited the growth of *Staphylococcus aureus*, and that this inhibition was abolished by L-glutamate. Inhibitions by commercial preparations of L-glutamine had been observed in this laboratory some time ago during work on streptococci, but no inhibition occurred when pure glutamine was used. Two strains of *Staphylococcus aureus* showed results similar to those of Grossowicz when a preparation of L-glutamine from The British Drug Houses (Batch 639261) was used. This specimen was purified by passing H_2S through a slightly alkaline solution and then treating it according to Archibald.[2] Concentrations up to 8.0 mg. per ml. of the purified material did not inhibit growth. The British Drug Houses examined the above batch of impure glutamine polarographically, and found 0.75 per cent Zn and 0.44 per cent Ba. The amide nitrogen was 54 per cent of the theoretical value. Other impurities

Growth of *Staphylococcus aureus* on the medium of Lichtenstein and Grossowicz* at 37°. Inoculum 10^4 organisms from 16 hour broth culture. The L-glutamate and L-glutamine were sterilized by filtration of a 1 per cent stock solution. 0 indicates no growth, + visible growth, ++++ maximum growth.

Substances added to 5 ml. medium	Growth		
	16 hrs.	24 hrs.	48 hrs.
None	+	++	++++
L-Glutamine (British Drug Houses), 10 mg.	0	0?	++++
" (" " " purified), 2–40 mg.	+	+++	++++
L-Glutamate, 2 mg.	0?	++	++++
Zn (as $ZnCl_2$), 0.001 mg.	0	0	0
" " " 0.005 "	0	0	0?
" " " 0.001 " L-glutamate, 2 mg.	0	++	++++
" " " 0.005 " " 2 "	0	+++	++++
" " " 0.01 " L-glutamine, 10 mg.	0	++	++++
" " " 0.005 " " 10 "	0?	+++	++++

* Lichtenstein, N., and Grossowicz, N., *J. Biol. Chem.*, **171**, 387 (1947).

---

[1] Grossowicz, N., *J. Biol. Chem.*, **173**, 729 (1948).

[2] Archibald, R. M., *J. Biol. Chem.*, **159**, 693 (1945).

were arginine (2 per cent), ammonium pyrrolidone carboxylate, and ammonium glutamate. Zn in concentrations comparable to those present in impure glutamine inhibited growth completely; this inhibition was reversed by L-glutamate and L-glutamine (see the table).

As Grossowicz used commercial preparations of glutamine, one of which was supplied by The British Drug Houses, it is probable that the inhibitions observed by him were due to impurities.

Medical Research Council Unit for Cell Metabolism
Department of Biochemistry
The University
Sheffield 10, England

D. E. Hughes

Received for publication, October 26, 1948

THE PARTICIPATION OF INORGANIC PYROPHOSPHATE IN THE REVERSIBLE ENZYMATIC SYNTHESIS OF DIPHOSPHOPYRIDINE NUCLEOTIDE

Sirs:

A purified enzyme preparation has been obtained from an autolysate of dried brewers' yeast by ammonium sulfate fractionation and isoelectric precipitation which catalyzes the reaction: nicotinamide mononucleotide (NMN) + adenosine triphosphate (ATP) ⇄ DPN + inorganic pyrophosphate (P-P). In the table are summarized two experiments in which equilibrium was attained starting from the left (DPN synthesis) and the right (ATP synthesis). The equilibrium constant, $K = ((\mathrm{DPN})(\mathrm{P\text{-}P}))/((\mathrm{NMN})(\mathrm{ATP}))$, calculated from the data of the two experiments is 0.3 in one case and 0.5 in the other. The concentrations of acid-labile phosphate were unchanged and no orthophosphate was produced. Nicotinamide nu-

Substance estimated†	DPN synthesis,* micromoles per ml.			ATP synthesis,* micromoles per ml.		
	0 min.	60 min.	Δ	0 min.	60 min.	Δ
NMN‡	2.50			0.0		
ATP	2.20	1.36	−0.84	0.0	1.02	+1.02
DPN	0.0	0.74	+0.74	1.50	0.61	−0.89
P-P	0.0	0.83	+0.83	1.80	0.79	−1.01
Orthophosphate	0.11	0.13		0.16	0.09	
Phosphate, acid-labile	4.44	4.50		3.60	3.66	

* For DPN synthesis, 1.0 ml. of reaction mixture contained 50 γ of the enzyme preparation, 0.3 micromole of $MgCl_2$, and 50 micromoles of glycylglycine buffer (pH 7.4) in addition to ATP and NMN; for ATP synthesis 1.0 ml. contained 50 γ of the enzyme preparation, 0.75 micromole of $MgCl_2$, and 50 micromoles of glycylglycine buffer (pH 7.4) in addition to DPN and P-P. Constant values were reached after 30 to 40 minutes at 38°.

† ATP was estimated spectrophotometrically by triphosphopyridine nucleotide reduction in the presence of glucose, hexokinase, and *Zwischenferment*, and DPN by reduction with the triose phosphate dehydrogenase system. Inorganic pyrophosphate was estimated as orthophosphate after acid hydrolysis of the precipitated and washed manganous salt and acid-labile phosphate as the orthophosphate released after 10 minute hydrolysis in 1 N H_2SO_4 at 100°.

‡ NMN was prepared by hydrolysis of DPN with nucleotide pyrophosphatase.[1] [2] After purification the ratio, nicotinamide-ribose moiety to organic phosphate, was 1.0.

---

[1] Kornberg, A., *J. Biol. Chem.*, **174**, 1051 (1948).

[2] Kornberg, A., and Lindberg, O., *J. Biol. Chem.*, **176**, 665 (1948).

cleoside, adenosine diphosphate, and adenylic acid were inactive in DPN synthesis. The reduced form of DPN was split by the purified enzyme preparation in the presence of inorganic pyrophosphate, but triphosphopyridine nucleotide and flavin-adenine dinucleotide were not. The possibility that the latter two nucleotides may participate in analogous reactions with crude enzyme preparations requires further study.

These findings indicate a mechanism for the synthesis of DPN and for the origin and function of inorganic pyrophosphate. Ochoa, Cori, and Cori[3] isolated inorganic pyrophosphate from dialyzed rat liver dispersions in which glutamate, pyruvate, or succinate was being oxidized. It was later identified in washed rabbit kidney particles oxidizing glutamate,[4,2] in molds,[5] and in yeast.[6] The present findings suggest that the accumulation of inorganic pyrophosphate in fungi and in tissues may be explained by a sequence of three reactions: (1) the irreversible hydrolysis of DPN by nucleotide pyrophosphatase[1,2] to yield NMN and adenylic acid, (2) the phosphorylation of adenylic acid to ATP in respiration or fermentation, and (3) the combination of NMN with ATP to produce inorganic pyrophosphate and regenerate DPN.

National Institutes of Health
Bethesda
Maryland

ARTHUR KORNBERG[7]

Received for publication, November 10, 1948

---

[3] Cori, C. F., in A symposium on respiratory enzymes, Madison (1942).

[4] Green, D. E., *et al.*, Abstracts, American Chemical Society, Atlantic City, 26B, April (1947).

[5] Mann, T., *Biochem. J.*, **38**, 345 (1944).

[6] Lindahl, P. E., and Lindberg, O., *Nature*, **157**, 335 (1946).

[7] The valuable technical assistance of Mr. W. E. Pricer, Jr., is gratefully acknowledged.

RELATION OF VITAMIN B_{12} TO THE GROWTH FACTOR PRESENT IN COW MANURE

Sirs:

Crystalline vitamin B_{12}, reported by Ott *et al.*[1] to have activity for chick growth, has been found to be completely effective, either orally or by intramuscular injection, when tested by the method developed in this laboratory[2] to assay quantities of the unknown growth factor occurring in cow manure, in fish-meal, and in some other feedstuffs of animal origin. Details of the method were the same as those reported previously,[2] except that the progeny of undepleted hens were used in the second of the two experiments. Injections were made into the breast muscle on the 1st day of the 14 day experimental period.

Experiment No.	Supplement	Level of supplement in diet, γ per 100 gm.	No. of chicks	Average gain, 2 to 4 wks. of age
				gm.
1	None		9	40.6
	Acid ppt.	75,000	9	77.3
	" "	150,000	8	89.1
	" "	300,000	8	94.9
	Vitamin B_{12} (crystalline)*	0.25	9	62.6
	" " "	0.50	9	81.7
	" " "	1.00	8	85.4
	" " "	2.00	9	100.9
		Single injection of supplement, γ per chick		
2	None		8	89.3
	2 unit liver extract†	100,000	9	98.3
	2 " " "	200,000	8	115.0
	2 " " "	300,000	9	116.6
	Vitamin B_{12} (crystalline)	1.25	9	123.4
	" " "	2.5	9	123.3
	" " "	5.0	9	120.4

* Prepared by Merck and Company, Inc., Rahway, New Jersey.
† Liver injection (crude), 2 U. S. P. units per ml.

The results in the table show that the maximum growth response was the same in Experiment 1 for crystalline vitamin B_{12} and the acid precipitate of water extract of cow manure, and essentially the same in Experiment 2 for the crystalline vitamin and 2 unit liver extract. The gains obtained in Experiment 1 were plotted against the dietary level of the crystalline vitamin and a dose-response curve constructed. The gain obtained with the lowest level of acid precipitate was applied to the straight line portion of the curve, and it was calculated that the acid precipitate contained the equivalent of 5.8 γ of vitamin B_{12}. Comparative chick and bacterial assays would be of interest as a means of determining the possible existence of different forms of the vitamin.

---

[1] Ott, W. H., Rickes, E. L., and Wood, T. R., *J. Biol. Chem.*, **174**, 1047 (1948).

[2] Bird, H. R., Rubin, M., and Groschke, A. C., *J. Biol. Chem.*. **174**, 611 (1948).

In view of the potency of this vitamin as a bacterial growth factor the injection experiments are of particular interest since they show that its effect on the chick is direct and not mediated through the intestinal flora.

Bureau of Animal Industry
Agricultural Research Administration
United States Department of Agriculture
Beltsville, Maryland

Robert J. Lillie
Charles A. Denton
H. R. Bird

Received for publication, October 23, 1948

INDEX TO AUTHORS

A

Åberg, Bertil. See *Jorpes, Werner, and Åberg,* 277

Abraham, Edward P. See *Heidelberger, Abraham, and Lepkovsky,* 1461

Adler, M., and **Wintersteiner, O.** A reinvestigation of flavacidin, the penicillin produced by *Aspergillus flavus,* 873

Albanese, Anthony A., Davis, Virginia I., Lein, Marilyn, and **Smetak, Emilie M.** The biochemistry of the metabolic fecal protein nitrogen, 1189

Aldous, J. G. The effect of pH upon the toxicity of iodoacetic acid to yeast cells, 83

Altman, Kurt I., Casarett, G. W., Masters, R. E., Noonan, T. R., and **Salomon, K.** Hemoglobin synthesis from glycine labeled with radioactive carbon in its α-carbon atom, 319

Anderson, Norman G. See *Wilbur and Anderson,* 147

Anfinsen, Christian B. See *Beloff and Anfinsen,* 863

Anker, H. S. Some aspects of the metabolism of pyruvic acid in the intact animal, 1337

—. Synthesis of carbonyl-labeled pyruvic acid, 1333

— and **Raper, R.** On the metabolic fate of pyruvamide and acetamide, 1353

Artom, Camillo, and **Cornatzer, W. E.** The action of ethanolamine, methylethanolamine, and dimethylethanolamine on lipide phosphorylation, 949

Atchley, William A. Studies on the cyclophorase system. IV. Direct demonstration of β oxidation, 123

Auerbach, V. H. See *Knox, Noyce, and Auerbach,* 117

Axelrod, A. E., Purvis, Sarah Ellen, and **Hofmann, Klaus.** The application of structural analogues to enzymatic studies. I. Studies on the mode of action of biotin analogues, 695

Axelrod, A. E. See *McCoy, McKibben, Axelrod, and Hofmann,* 1319, 1327

Axelrod, Bernard. A study of the mechanism of "phosphotransferase" activity by use of radioactive phosphorus, 295

Axelrod, Dorothy J. See *Scott, Axelrod, Fisher, Crowley, and Hamilton,* 283

B

Balls, A. K. See *Schwimmer and Balls,* 465

Barry, Guy T. See *Hogeboom and Barry,* 935

Bartlett, Mary N. See *Kochakian and Bartlett,* 243

Baumann, C. A. See *Sauberlich and Baumann,* 165

Beck, Jay V. A microbiological method for the determination of adenine, 1169

Beckman, William W. See *Hiller, Greif, and Beckman,* 1421

Behrens, Otto K., and **Kingkade, Mary Jane.** Biosynthesis of penicillins. VIII. Studies with new biosynthetic penicillins on penicillin resistance, 1047

Beloff, Anne, and **Anfinsen, Christian B.** The products of proteolysis of some purified proteins, 863

Bendich, Aaron, and **Brown, George Bosworth.** 2,6-Diaminopurine, a precursor of nucleic acid guanine, 1471

Bennett, Edward L., and **Niemann, Carl.** The use of ion exchange resins in the isolation of blood group A-specific substance from hog gastric mucin, 969

Bernheim, Frederick. See *Eadie, Bernheim, and Fitzgerald,* 857

Bird, H. R. See *Lillie, Denton, and Bird,* 1477

Bliss, Alfred F. The absorption spectra of visual purple of the squid and its bleaching products, 563

Blonstein, Muriel. See *Kibrick and Blonstein,* 983

Blum, Amos E. See *Horn, Jones, and Blum,* 59, 679

Borsook, Henry, Deasy, Clara L., Haagen-Smit, A. J., Keighley, Geoffrey, and Lowy, Peter H. The degradation of α-aminoadipic acid in guinia pig liver homogenate, 1395

—, —, —, —, and —. The degradation of L-lysine in guinea pig liver homogenate: formation of α-aminoadipic acid, 1383

—. See *Dubnoff and Borsook,* 789

Bosshardt, David K. See *Skeggs, Huff, Wright, and Bosshardt,* 1459

Bowen, William J. Notes on myoglobin preparation and iron content, 747

Brickson, W. L., Henderson, L. M., Solhjell, I., and Elvehjem, C. A. Antagonism of amino acids in the growth of lactic acid bacteria, 517

Briggs, G. M. See *Shorb and Briggs,* 1463

Broach, Wilson J. See *Keith, Broach, Warren, Day, and Totter,* 1095

Brown, Douglas M. See *Cartwright, Smith, Brown, and Wintrobe,* 585

—. See *Jager, Smith, Nickerson, and Brown,* 1177

Brown, Ellis V. See *Salivar, Grenfell, and Brown,* 977

Brown, George Bosworth. See *Bendich and Brown,* 1471

Bucciero, Mary C. See *Orten and Bucciero,* 961

Burkitt, Vonnie. See *Witter and Stotz,* 501

Burris, R. H. See *MacVicar and Burris,* 511

C

Caldwell, Alvin. See *Jones, Caldwell, and Horn,* 65

Cappiello, Marjorie. See *Wilhelmi, Fishman, and Russell,* 735

Carpenter, Frederick H., Stacy, Gardner W., Genghof, Dorothy S., Livermore, Arthur H., and du Vigneaud, Vincent. The preparation and antibacterial properties of the crude sodium salts of some synthetic penicillins, 915

Carpenter, Frederick H., Turner, Robert A., and du Vigneaud, Vincent. Benzylpenicillenic acid as an intermediate in the synthesis of benzylpenicillin (penicillin G), 893

Carroll, Richard P. See *Lester and Greenberg,* 53

Cartwright, George E., Smith, Emil L., Brown, Douglas M., and Wintrobe, Maxwell M. Electrophoretic analyses of sera of normal and hypoproteinemic swine, 585

— and **Wintrobe, Maxwell M.** Studies on free erythrocyte protoporphyrin, plasma copper, and plasma iron in protein-deficient and iron-deficient swine, 571

Casarett, G. W. See *Altman, Casarett, Masters, Noonan, and Salomon,* 319

Cerceo, Emily. See *Pearlman and Cerceo,* 847

Chaikoff, I. L. See *Srere, Chaikoff, and Dauben,* 829

—. See *Taurog and Chaikoff,* 639

—. See *Zilversmit, Chaikoff, Feller, and Masoro,* 389

—. See *Zilversmit, Entenman, and Chaikoff,* 193, 209

Chanutin, Alfred, and Ludewig, Stephan. Biochemical studies on the whole and fractionated thymus of rats injected with β-chloroethyl vesicants, 999

Chargaff, Erwin. See *Vischer and Chargaff,* 703, 715

Cheldelin, Vernon H. See *Fels and Cheldelin,* 819

Chinard, Francis P. Interactions of quaternary ammonium compounds and proteins. A simple method for the rapid estimation of urinary protein concentrations with alkyldimethyl-benzylammonium compounds, 1439

—. Use of the hypobromite reaction fo the estimation of ammonia plus ure nitrogen in urines containing larg amounts of protein; the reaction o alkaline hypobromite with proteins 144

Chipman, June. See *Geyer, Chipma and Stare,* 14

Cohen, Philip P. See *Grisolia and Cohen,* 929

Coolidge, Thomas B. See *Lightfoot and Coolidge,* 477

Cornatzer, W. E. See *Artom and Cornatzer,* 949

Crowley, J. F. See *Scott, Axelrod, Fisher, Crowley, and Hamilton,* 283

D

Dauben, W. G. See *Srere, Chaikoff, and Dauben,* 829

Davis, Virginia I. See *Albanese, Davis, Lein, and Smetak,* 1189

Dawson, Rex M. C. See *Richter and Dawson,* 1199

Day, Paul L. See *Keith, Broach, Warren, Day, and Totter,* 1095

Deasy, Clara L. See *Borsook, Deasy, Haagen-Smit, Keighley, and Lowy,* 1383, 1395

Denton, Charles A. See *Lillie, Denton, and Bird,* 1477

Deutsch, H. F., and **Nichol, J. C.** Biophysical studies of blood plasma proteins. X. Fractionation studies of normal and immune horse serum, 797

Dubnoff, Jacob W., and **Borsook, Henry.** Dimethylthetin and dimethyl-β-propiothetin in methionine synthesis, 789

E

Eadie, G. S., Bernheim, Frederick, and **Fitzgerald, Robert J.** The kinetics of the oxidation of benzoic acid by certain *Mycobacteria,* 857

Elvehjem, C. A. See *Brickson, Henderson, Solhjell, and Elvehjem,* 517

—. See *Hankes, Riesen, Henderson, and Elvehjem,* 467

Entenman, C. See *Zilversmit, Entenman, and Chaikoff,* 193, 209

Evans, Herbert M. See *Li, Simpson, and Evans,* 843

F

Farlow, M. W. A new synthesis of cystine, 71

Feller, D. D. See *Zilversmit, Chaikoff, Feller, and Masoro,* 389

Fels, I. Gordon, and **Cheldelin, Vernon H.** Methionine in selenium poisoning, 819

Fieser, Louis F., and **Heymann, Hans.** Naphthoquinone antimalarials. XXII. Relative antirespiratory activities (*Plasmodium lophurae*), 1363

—. See *Heymann and Fieser,* 1359

Fisher, Harvey. See *Scott, Axelrod, Fisher, Crowley, and Hamilton,* 283

Fishman, Jacob B. See *Wilhelmi, Fishman, and Russell,* 735

Fitzgerald, Robert J. The inactivation of streptomycin by cyanate, 223

—. See *Eadie, Bernheim, and Fitzgerald,* 857

Follett, Archie E. Preparation and some properties of hyaluronic acid from umbilical cord of the pig, 177

Franklin, A. L. See *Hoffmann, Stokstad, Franklin, and Jukes,* 1465

Frieden, Earl, and **Winzler, Richard J.** The thyroxine-like activity of compounds structurally related to thyroxine, 155

G

Gallagher, T. F. See *Munson, Jones, McCall, and Gallagher,* 73

Gardell, Sven. See *Jorpes and Gardell,* 267

Genghof, Dorothy S. See *Carpenter, Stacy, Genghof, Livermore, and du Vigneaud,* 915

Gest, Howard, and **Kamen, Martin D.** Studies on the phosphorus metabolism of green algae and purple bacteria in relation to photosynthesis, 299

Geyer, Robert P., Chipman, June, and **Stare, F. J.** Oxidation *in vivo* of emulsified radioactive trilaurin administered intravenously, 1469

Gibson, John G., 2nd. See *Vallee and Gibson,* 435, 445

Grafflin, Allan L., and **Green, D. E.** Studies on the cyclophorase system. II. The complete oxidation of fatty acids, 95

Green, Arda Alden. See *Rapport, Green, and Page,* 1237, 1243

Green, D. E. See *Grafflin and Green,* 95

Greenberg, Leon A. See *Lester and Greenberg,* 53

Greif, Roger L. See *Hiller, Greif, and Beckman,* 1421

Grenfell, Thomas C. See *Salivar, Grenfell, and Brown,* 977

Griffin, A. Clark, Nye, William N., Noda, Lafayette, and **Luck, J. Murray.** Tissue proteins and carcinogenesis. I. The effect of carcinogenic azo dyes on liver proteins, 1225

Grisolia, Santiago, and **Cohen, Philip P.** Study of carbon dioxide fixation in the synthesis of citrulline, 929

Grollman, Arthur. See *Stimmel, Grollman, and Huffman,* 461

Gunsalus, I. C. See *Umbreit, O'Kane, and Gunsalus,* 629

H

Haagen-Smit, A. J. See *Borsook, Deasy, Haagen-Smit, Keighley, and Lowy,* 1383, 1395

Hamilton, Joseph G. See *Scott, Axelrod, Fisher, Crowley, and Hamilton,* 283

Hankes, L. V., Riesen, W. H., Henderson, L. M., and **Elvehjem, C. A.** Liberation of amino acids from raw and heated casein by acid and enzyme hydrolysis, 467

Hanok, Albert. See *Sobel and Hanok,* 1103

Hanson, H. Theo. See *Smith and Hanson,* 997

Harding W. M. See *Shive, Ravel, and Harding,* 991

Harkness, David M. See *Seifter, Harkness, Rubin, and Muntwyler,* 1371

Harmon, Kent M. See *Peterson, Harmon, and Niemann,* 1

Heidelberger, Charles, Abraham, Edward P., and **Lepkovsky, Samuel.** Concerning the mechanism of the mammalian conversion of tryptophan into nicotinic acid, 1461

Henderson, L. M. See *Brickson, Henderson, Solhjell, and Elvehjem,* 517

—. See *Hankes, Riesen, Henderson, and Elvehjem,* 467

Heppel, Leon A., and **Porterfield, Virginia T.** Enzymatic dehalogenation of certain brominated and chlorinated compounds, 763

Herbst, Edward J., and **Snell, Esmond E.** Putrescine as a growth factor for *Hemophilus parainfluenzae,* 989

Heymann, Hans, and **Fieser, Louis F.** Naphthoquinone antimalarials. XXI. Antisuccinate oxidase activity, 1359

—. See *Fieser and Heymann,* 1363

Hiller, Alma, Greif, Roger L., and **Beckman, William W.** Determination of protein in urine by the biuret method, 1421

—, **Plazin, John,** and **Van Slyke, Donalc D.** A study of conditions for Kjeldah determination of nitrogen in proteins Description of methods with mercur as catalyst, and titrimetric and gaso metric measurements of the ammoni formed, 140

—, —, and —. Substitutes for saponi in the determination of oxygen an carbon monoxide of blood, 14

Hoffmann, C. E., Stokstad, E. L. R Franklin, A. L., and **Jukes, Thom H.** Response of *Lactobacillus leic mannii* 313 to the antipernicio anemia factor, 14

Hofmann, Klaus. See *Axelrod, Purv and Hofmann,*

—. See *McCoy, McKibben, Axelrod, a Hofmann,* 1319, 1

Hogeboom, George H., and **Barry, G T.** Purification of diphosphopyrid nucleotide by counter-current distri tion,

Hogness, John R. See *Roth, Lei Hogness, and Langham,*

Horn, Millard J., Jones, D. Breese, Blum, Amos E. Microbiological termination of arginine in proteins foods,

—, —, and —. Microbiological de

mination of phenylalanine in proteins and foods, 679

Horn, Millard J. See *Jones, Caldwell, and Horn,* 65

Huff, Jesse W. See *Skeggs, Huff, Wright, and Bosshardt,* 1459

Huffman, Max N. See *Stimmel, Grollman, and Huffman,* 461

Hughes, D. E. Glutamine and the growth of *Staphylococcus aureus,* 1473

J

Jager, B. V., Smith, Emil L., Nickerson, Margaret, and **Brown, Douglas M.** Immunological and electrophoretic studies on human γ-globulins, 1177

Jansen, Eugene F. The isolation and identification of 2,2′-dithiolisobutyric acid from asparagus, 657

Jeanes, Allene, Schieltz, N. C., and **Wilham, C. A.** Molecular association in dextran and in branched amylaceous carbohydrates, 617

—, **Wilham, C. A.,** and **Miers, J. C.** Preparation and characterization of dextran from *Leuconostoc mesenteroides,* 603

Jones, D. Breese, Caldwell, Alvin, and **Horn, Millard J.** The availability of DL-lanthionine for the promotion of growth in young rats when added to a cystine- and methionine-deficient diet, 65

—. See *Horn, Jones, and Blum,* 59, 679

Jones, Mary Ellen. See *Munson, Jones, McCall, and Gallagher,* 73

Jorpes, J. Erik, and **Gardell, Sven.** On heparin monosulfuric acid, 267

—, **Werner, Birgitta,** and **Åberg, Bertil.** The fuchsin-sulfurous acid test after periodate oxidation of heparin and allied polysaccharides, 277

Jukes, Thomas H. See *Hoffmann, Stokstad, Franklin, and Jukes,* 1465

K

Kamen, Martin D. See *Gest and Kamen,* 299

Kaplan, Nathan O., and **Lipmann, Fritz.** The acetyl precursor in pyruvate synthesis in *Escherichia coli,* 459

Keighley, Geoffrey. See *Borsook, Deasy, Haagen-Smit, Keighley, and Lowy,* 1383, 1395

Keith, Cecilia K., Broach, Wilson J., Warren, Doris, Day, Paul L., and **Totter, John R.** Xanthine oxidase and tyrosinase in the livers of chicks receiving graded levels of dietary pteroylglutamic acid, 1095

Kibrick, Andre C., and **Blonstein, Muriel.** Fractionation of serum into albumin and α-, β-, and γ-globulin by sodium sulfate, 983

Kielley, W. Wayne, and **Meyerhof, Otto.** Studies on adenosinetriphosphatase of muscle. II. A new magnesium-activated adenosinetriphosphatase, 591

King, C. G. See *Prusoff, Teply, and King,* 1309

Kingkade, Mary Jane. See *Behrens and Kingkade,* 1047

Kirsner, Joseph B. See *Sheffner, Kirsner, and Palmer,* 89

Knox, W. Eugene, Noyce, B. N., and **Auerbach, V. H.** Studies on the cyclophorase system. III. Obligatory sparking of fatty acid oxidation, 117

Kochakian, Charles D., and **Bartlett, Mary N.** The effect of crystalline adrenal cortical steroids, DL-thyroxine, and epinephrine on the alkaline and acid phosphatases and arginase of the liver and kidney of the normal adult rat, 243

Komarov, S. A. See *Siplet, Komarov, and Shay,* 545

Korkes, Seymour, and **Ochoa, Severo.** Adaptive conversion of malate to lactate and carbon dioxide by *Lactobacillus arabinosus,* 463

Kornberg, Arthur. The participation of inorganic pyrophosphate in the reversible enzymatic synthesis of diphosphopyridine nucleotide, 1475

— and **Lindberg, Olov.** Diphosphopyridine nucleotide pyrophosphatase, 665

Krishnaswamy, K. G., and **Sreenivasan, A.** Separation and determination of the amylose and amylopectin fractions of starch, 1253

L

Lamanna, Carl. See *Putnam, Lamanna, and Sharp,* 401

Lamb, Catherine M. See *Petermann and Lamb,* 685

Langham, Wright H. See *Roth, Leifer, Hogness, and Langham,* 249

Leathem, James H. Influence of testosterone propionate on the plasma and liver proteins of hypothyroid rats, 1285

Leifer, Edgar. See *Roth, Leifer, Hogness, and Langham,* 249

Lein, Marilyn. See *Albanese, Davis, Lein, and Smetak,* 1189

LePage, G. A. Glycolysis in tumor homogenates, 1009

— and **Schneider, Walter C.** Centrifugal fractionation of glycolytic enzymes in tissue homogenates, 1021

Lepkovsky, Samuel. See *Heidelberger, Abraham, and Lepkovsky,* 1461

Lester, David, and **Greenberg, Leon A.** A simple apparatus for the serial measurement of the respiratory exchange in the rat, 53

Levedahl, Blaine H., and **Samuels, Leo T.** The effect of testosterone and methyltestosterone on guanidoacetic acid, creatine, and creatinine in plasma and urine, 327

Levy, Milton, and **Young, Nelson F.** Chemistry of the chick embryo. VI. Accumulation of diphosphopyridine nucleotide, 185

Li, Choh Hao, Simpson, Miriam E., and **Evans, Herbert M.** Iodination of hypophyseal growth hormone, 843

Lifson, Nathan, Lorber, Victor, Sakami, Warwick, and **Wood, Harland G.** The incorporation of acetate and butyrate carbon into rat liver glycogen by pathways other than carbon dioxide fixation, 1263

Lightfoot, Lois H., and **Coolidge, Thomas B.** The distribution of collagen in the guinea pig, 477

Lillie, Robert J., Denton, Charles A., and **Bird, H. R.** Relation of vitamin B_{12} to the growth factor present in cow manure, 1477

Lindberg, Olov. See *Kornberg and Lindberg,* 665

Lipmann, Fritz. See *Kaplan and Lipmann,* 459

Litt, Irene. See *Mazur and Shorr,* 771

Littlejohn, Julia M. See *Singal, Sydenstricker, and Littlejohn,* 1051, 1063, 1069

Livermore, Arthur H. See *Carpenter, Stacy, Genghof, Livermore, and du Vigneaud,* 915

Lorber, Victor. See *Lifson, Lorber, Sakami, and Wood,* 1263

Loring, Hubert S., Ordway, George L., and **Pierce, John G.** A method of assay for cytidine and uridine by means of a pyrimidine-deficient strain of *Neurospora,* 1123

—. See *Pierce and Loring,* 1131

Lowy, Peter H. See *Borsook, Deasy, Haagen-Smit, Keighley, and Lowy,* 1383, 1395

Luck, J. Murray. See *Griffin, Nye, Noda, and Luck,* 1225

Ludewig, Stephan. See *Chanutin and Ludewig,* 999

Lyle, Gloria G. See *Pardee and Potter,* 1085

—. See *Potter, Pardee, and Lyle,* 1075

M

MacFarland, M. L., and **McHenry, E. W.** Further observations on the lipotropic need for inositol, 429

MacLeod, Robert A., and **Snell, Esmond E.** The effect of related ions on the potassium requirement of lactic acid bacteria, 39

MacVicar, Robert, and **Burris, R. H.** Studies on nitrogen metabolism in tomato with use of isotopically labeled ammonium sulfate, 511

Masoro, E. J. See *Zilversmit, Chaikoff, Feller, and Masoro,* 389

Masters, R. E. See *Altman, Casarett, Masters, Noonan, and Salomon,* 319

Mathews, Martin B. The reduction of cozymase by sodium borohydride, 229

Mattson, Fred H. The site of conversion of carotene to vitamin A, 1467

Maw, George A., and du Vigneaud, Vincent. Compounds related to dimethylthetin as sources of labile methyl groups, 1037

— and —. An investigation of the biological behavior of the sulfur analogue of choline, 1029

Mazur, Abraham, and Shorr, Ephraim. Hepatorenal factors in circulatory homeostasis. IX. The identification of the hepatic vasodepressor substance, VDM, with ferritin, 771

McCall, Philip J. See *Munson, Jones, McCall, and Gallagher,* 73

McCoy, R. H., McKibben, Juliet N., Axelrod, A. E., and Hofmann, Klaus. Oxybiotin metabolism in the chick. I. Deposition of oxybiotin in tissues, 1319

—, —, —, and —. II. Oxybiotin and biotin balance studies, 1327

McHenry, E. W. See *MacFarland and McHenry,* 429

McKibben, Juliet N. See *McCoy, McKibben, Axelrod, and Hofmann,* 1319, 1327

Meyer, Karl. Highly viscous sodium hyaluronate, 993

Meyerhof, Otto. See *Kielley and Meyerhof,* 591

Miers, J. C. See *Jeanes, Wilham, and Miers,* 603

Miller, J. A. See *Mueller and Miller,* 535

Milner, Harold W. The fatty acids of *Chlorella,* 813

Moore, Stanford, and Stein, William H. Photometric ninhydrin method for use in the chromatography of amino acids, 367

—. See *Stein and Moore,* 337

Moubasher, Radwan. Reaction of *peri*-naphthindan-2,3,4-trione hydrate with *l*-ascorbic acid and other enediol compounds, with a note on the isolation of dehydro-*l*-ascorbic acid, 529

Mueller, G. C., and Miller, J. A. The metabolism of 4-dimethylaminoazobenzene by rat liver homogenates, 535

Munson, Paul L., Jones, Mary Ellen, McCall, Philip J., and Gallagher, T. F. A colorimetric method for estimation of dehydroisoandrosterone and its application to urine extracts, 73

Muntwyler, Edward. See *Seifter, Harkness, Rubin, and Muntwyler,* 1371

N

Newell, Dora M. See *Chinard,* 1439, 1449

Nichol, J. C. See *Deutsch and Nichol,* 797

Nickerson, Margaret. See *Jager, Smith, Nickerson, and Brown,* 1177

Niemann, Carl. See *Bennett and Niemann,* 969

—. See *Peterson, Harmon, and Niemann,* 1

Noda, Lafayette. See *Griffin, Nye, Noda, and Luck,* 1225

Noonan, T. R. See *Altman, Casarett, Masters, Noonan, and Salomon,* 319

Noyce, B. N. See *Knox, Noyce, and Auerbach,* 117

Nye, William N. See *Griffin, Nye, Noda, and Luck,* 1225

O

Ochoa, Severo. See *Korkes and Ochoa,* 463

Oesterling, M. Jane. See *Rose, Oesterling, and Womack,* 753

O'Kane, D. J. See *Umbreit, O'Kane, and Gunsalus,* 629

Ordway, George L. See *Loring, Ordway, and Pierce,* 1123

Orten, James M., and Bucciero, Mary C. The effect of cysteine, histidine, and methionine on the production of polycythemia by cobalt, 961

P

Page, Irvine H. See *Rapport, Green, and Page,* 1237, 1243

Palmer, Walter L. See *Sheffner, Kirsner, and Palmer,* 89

Pardee, Arthur B., and Potter, Van R. Inhibition of succinic dehydrogenase by oxalacetate, 1085

—. See *Potter, Pardee, and Lyle,* 1075

Pearlman, W. H., and **Cerceo, Emily.** The isolation of pregnanol-3(α)-one-20, pregnanediol-3(α),20(β), and etiocholanediol-3(α),17(β) from the bile of pregnant cows, 847

Petermann, Mary L., and **Lamb, Catherine M.** The nucleohistone of beef spleen, 685

Peterson, Jack, Harmon, Kent M., and **Niemann, Carl.** The dependence of the specific activity of urease upon the apparent absolute enzyme concentration, 1

Peterson, W. H. See *Thorne and Peterson,* 413

Pierce, John G., and **Loring, Hubert S.** Purine and pyrimidine antagonism in a pyrimidine-deficient mutant of *Neurospora,* 1131

—. See *Loring, Ordway, and Pierce,* 1123

Plazin, John. See *Hiller, Greif, and Beckman,* 1421

—. See *Hiller, Plazin, and Van Slyke,* 1401, 1431

Porterfield, Virginia T. See *Heppel and Porterfield,* 763

Potter, Van R., Pardee, Arthur B., and **Lyle, Gloria G.** The assay of animal tissues for respiratory enzymes. VIII. Conditions for oxalacetate oxidation in whole homogenates, 1075

—. See *Pardee and Potter,* 1085

Prusoff, W. H., Teply, L. J., and **King, C. G.** The influence of pteroylglutamic acid on nucleic acid synthesis in *Lactobacillus casei,* 1309

Purvis, Sarah Ellen. See *Axelrod, Purvis, and Hofmann,* 695

Putnam, Frank W., Lamanna, Carl, and **Sharp, D. G.** Physicochemical properties of crystalline *Clostridium botulinum* type A toxin, 401

R

Rabinowitz, Jesse C., and **Snell, Esmond E.** The vitamin B_6 group. XIV. Distribution of pyridoxal, pyridoxamine, and pyridoxine in some natural products, 1157

Raper, R. See *Anker and Raper,* 1353

Rapport, Maurice M., Green, Arda Alden, and **Page, Irvine H.** Serum vasoconstrictor (serotonin). III. Chemical inactivation, 1237

—, —, and —. IV. Isolation and characterization, 1243

Ravel, Joanne Macow. See *Shive, Ravel, and Harding,* 991

Richter, Derek, and **Dawson, Rex M. C.** The ammonia and glutamine content of the brain, 1199

Riesen, W. H. See *Hankes, Riesen, Henderson, and Elvehjem,* 467

Roberts, Eugene. Estimation of arginase activity in homogenates, 213

Rose, William C., Oesterling, M. Jane, and **Womack, Madelyn.** Comparative growth on diets containing ten and nineteen amino acids, with further observations upon the rôle of glutamic and aspartic acids, 753

Roth, Lloyd J., Leifer, Edgar, Hogness, John R., and **Langham, Wright H.** Studies on the metabolism of radioactive nicotinic acid and nicotinamide in mice, 249

Rubin, Leonard. See *Seifter, Harkness, Rubin, and Muntwyler,* 1371

Russell, Jane A. See *Wilhelmi, Fishman, and Russell,* 735

S

Sakami, Warwick. The conversion of formate and glycine to serine and glycogen in the intact rat, 995

—. See *Lifson, Lorber, Sakami, and Wood,* 1263

Salivar, Charles J., Grenfell, Thomas C., and **Brown, Ellis V.** Studies on the naturally occurring penicillins. II. Precipitation of crystalline ammonium penicillins, 977

Salomon, K. See *Altman, Casarett, Masters, Noonan, and Salomon,* 319

Samuels, Leo T. See *Levedahl and Samuels,* 327

Sauberlich, H. E., and **Baumann, C. A.** A factor required for the growth of *Leuconostoc citrovorum,* 165

Sayers, George. See *Wilhelmi and Sayers,* 175

Schieltz, N. C. See *Jeanes, Schieltz, and Wilham,* 617

Schneider, Walter C. Intracellular distribution of enzymes. III. The oxidation of octanoic acid by rat liver fractions, 259

—. See *LePage and Schneider,* 1021

Schwimmer, Sigmund, and **Balls, A. K.** Crystalline α-amylase from barley malt, 465

Scott, Kenneth G., Axelrod, Dorothy J., Fisher, Harvey, Crowley, J. F., and **Hamilton, Joseph G.** The metabolism of plutonium in rats following intramuscular injection, 283

Seifter, Sam, Harkness, David M., Rubin, Leonard, and **Muntwyler, Edward.** The nicotinic acid, riboflavin, D-amino acid oxidase, and arginase levels of the livers of rats on a protein-free diet, 1371

Sharp, D. G. See *Putnam, Lamanna, and Sharp,* 401

Shay, Harry. See *Siplet, Komarov, and Shay,* 545

Sheffner, A. Leonard, Kirsner, Joseph B., and **Palmer, Walter L.** Studies on amino acid excretion in man. II. Amino acids in feces, 89

Shive, William, Ravel, Joanne Macow, and **Harding, W. M.** An interrelationship of purines and vitamin B_{12}, 991

Shorb, Mary S., and **Briggs, G. M.** The effect of dissociation in *Lactobacillus lactis* cultures on the requirement for vitamin B_{12}, 1463

Shorr, Ephraim. See *Mazur and Shorr,* 771

Simpson, Miriam E. See *Li, Simpson, and Evans,* 843

Singal, S. A., Sydenstricker, V. P., and **Littlejohn, Julia M.** Further studies on the effect of some amino acids on the growth and nicotinic acid storage of rats on low casein diets, 1063

—, —, and —. The nicotinic acid content of tissues of rats on corn rations, 1069

—, —, and —. The rôle of tryptophan in the nutrition of dogs on nicotinic acid-deficient diets, 1051

Siplet, Herman, Komarov, S. A., and **Shay, Harry.** The estimation of mucin in gastric juice, 545

Skeggs, Helen R., Huff, Jesse W., Wright, Lemuel D., and **Bosshardt, David K.** The use of *Lactobacillus leichmannii* in the microbiological assay of the "animal protein factor," 1459

Slonim, N. Balfour. See *Smith and Slonim,* 835

Smetak, Emilie M. See *Albanese, Davis, Lein, and Smetak,* 1189

Smith, Emil L. Studies on dipeptidases. II. Some properties of the glycyl-L-leucine dipeptidases of animal tissues, 9

—. III. Hydrolysis of methylated peptides; the rôle of cobalt in the action of glycylglycine dipeptidase, 21

— and **Hanson, H. Theo.** Pancreatic carboxypeptidase; a metal protein, 997

— and **Slonim, N. Balfour.** The specificity of leucine aminopeptidase, 835

—. See *Cartwright, Smith, Brown, and Wintrobe,* 585

—. See *Jager, Smith, Nickerson, and Brown,* 1177

Snell, Esmond E. See *Herbst and Snell,* 989

—. See *MacLeod and Snell,* 39

—. See *Rabinowitz and Snell,* 1157

Snyder, Jeannette. See *Witter, Snyder, and Stotz,* 493

Sobel, Albert E., and **Hanok, Albert.** Calcification of teeth. I. Composition in relation to blood and diet, 1103

Solhjell, I. See *Brickson, Henderson, Solhjell, and Elvehjem,* 517

Sreenivasan, A. See *Krishnaswamy and Sreenivasan,* 1253

Srere, Paul A., Chaikoff, I. L., and **Dauben, W. G.** The *in vitro* synthesis of cholesterol from acetate by surviving adrenal cortical tissue, 829

Stacy, Gardner W. See *Carpenter, Stacy, Genghof, Livermore, and du Vigneaud,* 915

—. See *du Vigneaud, Stacy, and Todd,* 907